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THE ANALYSIS OF GENETIC-ENVIRONMENTAL INTERACTIONS BY MEANS OF DIALLEL CROSSES

R. W. ALLARD

University of California, Davis, California

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ALTHOUGH it is a matter of importance both theoretically and practically, little is known about the constancy under different environmental conditions of the genetic components of continuous variation. This is not surprising, for study of the effects of environment upon the various genetic parameters required to specify continuous variation could not proceed until the parameters themselves were clearly defined and techniques had been developed for their detection or measurement. Recently, as a result of an expansion and generalization of the method of analysis of diallel crosses, JINKS (1952, 1955) has shown that the various statistics obtained from measurements of parents and their F_1 progeny in diallel crosses provide estimates of overall dominance, the relative dominance of parents, and the distribution of dominant and recessive alleles in the parental lines entering the diallel system. Even more important, the analysis permits the separation of certain types of non-allelic interaction from overdominance. Further extensions of the analysis and applications to a variety of experimental data (JINKS and HAYMAN 1953; HAYMAN 1954b, 1955; JINKS 1954, 1955) have demonstrated the usefulness of diallel crosses in the investigation of certain of the genetic components of continuous variation.

Diallel crosses may also be useful in the investigation of genetic-environmental interactions, and it is with this aspect of the analysis of diallel experiments that the present discussion is concerned. It will be shown that estimates of stability under different environmental conditions of certain genetic parameters can be obtained from diallel experiments replicated in time or in space.

THE ANALYSIS OF DIALLEL CROSSES

The analysis of diallel crosses has been discussed in detail elsewhere (HULL 1945; GRIFFING 1950; JINKS 1952, 1954; JINKS and HAYMAN 1953; HAYMAN 1954a, 1954b) and need only be summarized here. The analysis depends upon the estimation of the genetic parameters D, H_1, H_2, and F as defined by JINKS (1954), who follows the notation of MATHER (1949). Mean dominance is measured by the ratio H_1/D, i.e., the ratio of the weighted sums of h^2 and d^2 where h and d are the dominance and additive components of variation, respectively. F indicates whether dominant or recessive alleles are more frequent in the parents, being positive if dominant alleles are in excess. $H_2/4H_1$ provides an estimate of the mean value of uv, where u is the proportion of positive alleles and v the proportion of negative alleles, and $u + v = 1$. It has a maximal value of $\frac{1}{4}$ when all $u = v = \frac{1}{2}$.

The regression of W_r on V_r (the covariances and variances of parental arrays) provides a geometric representation of the degree of dominance and also permits the separation of true dominance from the spurious "dominance" caused by many types of non-allelic interaction. With no dominance, the variances and covariances

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of arrays all estimate the point $(\bar{W}_r, \bar{V}_r) = (\frac{1}{2}D, \frac{1}{4}D)$ and there is, of course, no regression. With dominance, the regression line has unit slope. It intersects the W_r axis at the origin with complete dominance ($H_1 = D$), on the positive side with partial dominance ($H_1 < D$) and on the negative side with overdominance ($H_1 > D$). Most types of non-allelic interaction affect the regression line in a characteristic way, thus permitting their detection regardless of the degree of dominance. For example, complementary type of gene action increases V_r in relation to W_r, particularly for the more recessive arrays, leading to a regression line with slope less than unity.

The above relationships are not necessarily valid if (1) the inheritance is other than Mendelian and diploid, (2) the parental lines are heterozygous, (3) the genes are not independently distributed in the parents, and (4) more than two alleles exist at any one locus. The effects of non-validity of these assumptions have been considered by HAYMAN (1954b).

GENETIC-ENVIRONMENTAL INTERACTIONS

The fact that a standard error can be calculated for each of the above statistics makes possible the estimation of the significance of certain inter-environmental differences, e.g., whether mean dominance is the same in different seasons. However, a more general method of testing genetic-environmental interactions is possible from analysis of (1) the means and variances of the parents and (2) the variances and covariances of the arrays of the diallel table.

Heritable differences between the homozygous parents, in the absence of non-allelic interaction, must result from the additive effects of genes (D). Hence, parental lines differing significantly from each other must carry genes with different additive effects. Furthermore, constancy of the additive components of variation can be detected unambiguously by the parents $\times$ environments interaction item of an analysis of variance of parents. Significance provides evidence that they interact with environment, and non-significance is suggestive of the constancy of the additive effects under different environmental conditions. When non-allelic interactions are a feature of the genetic system, the situation is unambiguous if the parents $\times$ environments item is non-significant. If this item is significant, however, the conclusions cannot be clear-cut without evidence from analysis of the variances and covariances of the arrays of the diallel table because the significance could result from inconsistency of the additive effects, from inconsistency of the interactions between homozygous genes under the different environmental circumstances, or from both causes.

An analysis of variance of W_r and V_r from the diallel table provides further information about genetic-environmental interactions. But before this can be discussed, it is necessary to consider the genetic components of W_r and V_r. If a number of genes are independently distributed among several parental lines with alleles of positive and negative effect in the proportions u and v ($u + v = 1$), the covariance and variance of the rth array are

$$W_r = \Sigma\, 2\, uvd(d \pm h)$$

$$V_r = \Sigma\, uv(d \pm h)^2$$

where negative signs correspond to positive alleles in the rth parent and vice versa. The difference $W_r - V_r = \Sigma\, uv(d^2 - h^2) = \frac{1}{4}(D - H_1)$, which is independent of the signs of the alleles in the parents. Asymmetry of distribution of alleles of positive and negative effects does not upset the relationship between W_r and V_r, i.e., the points on the (W_r, V_r) graph lie on a straight line regardless of the state of symmetry.

W_r is made up of terms in d^2 and dh, while V_r is made up of these same terms plus additional terms in h^2. The relationship between W_r and V_r, therefore, varies only with the dominance components of variation. We have seen that a good test for the constancy of the additive components of variation is available from analysis of the parents. It is expedient, therefore, to minimize this component of variation in the analysis of W_r and V_r in order to improve the prospects of detecting interactions of dominance effects with environment. Dividing W_r and V_r by the variance of the parents ($V_p = D$) occurring in the same block accomplishes this result. This procedure has an additional advantage. It has been observed that basic variability fluctuates widley under different environments, tending to obscure between-environment comparisons of the genetic systems. Rescaling in the manner suggested alleviates this difficulty by placing the comparisons on similar scales.

Genetic significance of the components of variation

The genetic significance of the variances and covariances of arrays of a diallel experiment that has been replicated in time can be investigated by an analysis of variance with degrees of freedom as given in table 1. Two or more blocks of the diallel should be grown each year to permit error variance to be estimated from variation within years. The Years component of variation is calculated from the differences between the sums of $W_r + V_r$ over all arrays and blocks for each year. It therefore detects variation in $\bar{W}_r + \bar{V}_r$, i.e., $D + H_1 + F$ over years before rescaling or in $(D + H_1 + F)/D$ after rescaling. On the (W_r, V_r) graph the single points representing each year would therefore be expected to occupy the same position when environmental conditions under which the diallels were grown were identical. The Years component in the analysis of variance table should be nonsignificant under these circumstances. Significance of this component of variation can result from a change in response to environmental differences of one or any

TABLE 1

Components of variation and degrees of freedom for a diallel experiment replicated in time

Component of variation	Degrees of freedom
Years (environments)	$y - 1$*
Dominance	1
Years × Dominance	$y - 1$
Arrays	$a - 1$
Years × Arrays	$(y - 1)(a - 1)$
Dominance × Arrays	$a - 1$
Years × Dominance × Arrays	$(y - 1)(a - 1)$
Total	$2(y)(a) - 1$

* y and a denote the number of years (environments) and the number of arrays, respectively.

combination of D, H_1, or F in unrescaled data. Thus, in a favorable season $D + H_1 + F$ would be expected to be larger than in an unfavorable season even though the ratio of additive effects to dominance effects remained unchanged. Rescaling by dividing W_r and V_r by the variance of the parents should, however, minimize environmental effects of this type. The most likely causes of significance of the Years component of variation (after rescaling) are differences in mean dominance.

The Dominance component of variation is calculated from the difference between the sum of W_r and the sum of V_r over all years, blocks, and arrays. From diallel theory it is clear that the magnitude of this difference is governed by the degree of dominance, the differences $\bar{W}_r - \bar{V}_r$ providing a measure of $D - H_1$ (see table 5). The Dominance component of variation can therefore be expected to be significant except when mean dominance is complete, at which point $\bar{W}_r = \bar{V}_r$ and $D = H_1$. The Years × Dominance component of variation clearly provides an estimate of the constancy of mean dominance in different years. Thus, for example, if $D = H_1$ in one year and $D = \frac{1}{2}H_1$ in another year, both the Dominance and the Years × Dominance components will be significant, the former because $\bar{W}_r \neq \bar{V}_r$ on the average and the latter because dominance is inconsistent over the two seasons.

The Arrays component of variation is calculated from the sums of $\bar{W}_r + V_r$ for each array over all years and blocks. This component of variation detects differences in rank along the regression line, i.e., in the dominance order of the parents (see table 5). With no dominance the points representing arrays all occupy the same position $(W_r, V_r) = (\frac{1}{2}D, \frac{1}{4}D)$. If dominance exists, the points scatter along the regression line at positions indicating the relative dominance of the genes of the parents. The Arrays component of variation therefore provides a test of the significance of differences in dominance among the parents entering the diallel experiment. The constancy of the dominance relationships among different parents is clearly tested by the Years × Arrays component of variation. For example, if two genes of equal additive effect govern a character and $h_a > h_b$, the dominance order will be: (1) *AABB*, (2) *AAbb*, (3) *aaBB*, (4) *aabb*. If the dominance of the *Aa* gene pair is reversed by a change in environment, the order will no longer be 1 2 3 4 but becomes 3 4 1 2. Under these circumstances, the Arrays item in the analysis of variance would be significant because dominance is operative. The Years × Arrays item should also be significant because the relative dominance of the parents changed with environment.

The last two components of variation, Dominance × Arrays and Years × Dominance × Arrays, provide tests for non-allelic interaction and for the constancy of such effects over environments, respectively. These items will be considered in detail later.

NUMERICAL EXAMPLE

Data with which to test this type of analysis have been kindly supplied by Dr. J. L. Jinks. They consist of the flowering times from an arbitrary date of eight varieties of *Nicotiana rustica* and their $n^2 - n$ hybrids (including reciprocals), all grown in the experimental gardens of the University of Birmingham in 1951, 1952, and 1953. There were 64 plots each year. Each parent or hybrid was represented

TABLE 2

Variances and covariances of arrays for flowering time and height for 1951, 1952, and 1953 (× 10)

Array	Flowering time											
	1951				1952				1953			
	Block 1		Block 2		Block 1		Block 2		Block 1		Block 2	
	W	*V*	*W*	*V*	*W*	*V*	*W*	*V*	*W*	*V*	*W*	*V*
1	522	376	320	276	183	245	140	254	1107	906	1089	862
2	199	70	118	49	74	56	72	66	407	221	440	225
3	904	804	591	543	232	306	179	228	1213	1220	1288	1173
4	493	415	258	164	104	75	111	102	248	186	413	220
5	212	63	103	56	44	25	57	48	128	184	319	166
6	405	165	270	109	154	141	166	184	1191	1064	1261	1074
7	410	204	394	217	37	20	48	48	281	92	254	75
8	219	57	73	26	27	38	40	83	406	172	463	180
Mean variance of parents	1207		896		203		196		1480		1519	

Array	Height											
	1951				1952				1953			
	Block 1		Block 2		Block 1		Block 2		Block 1		Block 2	
	W	*V*	*W*	*V*	*W*	*V*	*W*	*V*	*W*	*V*	*W*	*V*
1	414	1060	554	900	578	1143	506	907	936	1930	1039	1712
2	221	276	106	146	160	337	53	158	245	896	315	886
3	350	433	506	544	529	745	536	708	736	1029	901	1148
4	37	405	68	336	−122	261	−69	118	−101	456	20	763
5	307	457	493	606	303	306	330	345	722	1060	989	1447
6	398	715	537	586	436	569	473	602	905	1842	1245	2433
7	213	335	438	475	297	223	374	426	637	877	695	729
8	282	371	488	562	316	294	456	552	720	1075	877	1066
Mean variance of parents	329		550		461		511		560		819	

by 10 plants, grown in two plots of 5 plants, with one plot in each of two blocks. Table 2 contains the variances and covariances of arrays of the diallel table (multiplied by 10) and the mean variances of the parents (also multiplied by 10). The data for each of the three years were first analyzed in the manner described by JINKS (1952, 1954) and HAYMAN (1954a). Before analysis, each variance and covariance was rescaled by dividing by the mean variance of the parents occurring in the same block.

(W_r, V_r) graphs calculated from these data are given in figure 1. A striking anomaly of these graphs is the behavior of arrays 1 and 3 in 1952. JINKS (1954) has explained this anomalous behavior on the basis of environmental effects associated with a planting procedure peculiar to that season. When disturbances of this sort occur, or if sporadic non-allelic interaction is encountered, the offending hybrids can be eliminated from the analysis by missing plot technique as suggested by HAYMAN

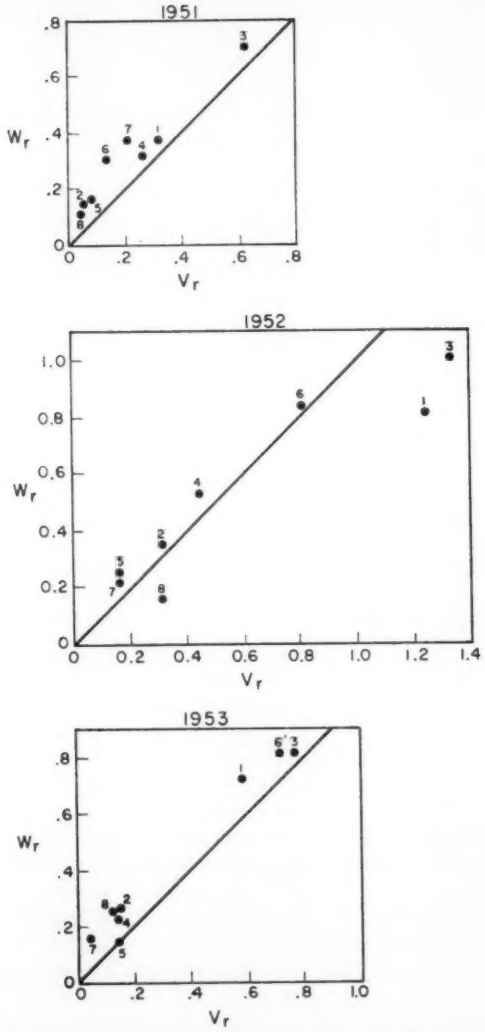

FIGURE 1.—(W_r, V_r) graphs for flowering time for 1951, 1952, and 1953. Points represent the position of parental arrays in relation to the regression line of unit slope through the origin. See text for interpretation.

TABLE 3

Analysis of variance of the mean flowering times for the eight parental varieties in 1951 and 1953

Component of variation	d.f.	Sums of squares	Variance ratio	Probability
Years	1	152.25	11.46	0.01–0.001
Parents	7	12,801.37	137.60	<0.001
Years × Parents	7	1,281.20	13.77	<0.001
Error	16	212.58		

(1954b). This procedure permits the remaining entries to be analyzed and the information from them to be salvaged. Of course, no information is obtained from the hybrids that were eliminated. Rather than complicate the present discussion by an adjustment of this sort, it should be sufficient for illustrative purposes to omit completely the 1952 data and focus attention on the results of 1951 and 1953.

The (W_r, V_r) graphs of figure 1 for those years reveal that the pattern of inheritance of flowering time is one of partial dominance uncomplicated by non-allelic interaction or non-validity of any of the assumptions stated earlier.

The first step in the investigation of genetic-environmental interactions between 1951 and 1953 consisted of an analysis of the means of the parents for the two years as shown in table 3. The significance of the years item has no specific genetical interpretation, for the planting dates were not the same in the two years. Indeed, even had the planting dates been the same, any of a host of environmental factors could have caused the observed difference in the mean flowering dates for the two seasons. The significance of the parents item, however, is direct evidence that certain parents (actually 5 out of 8) flowered at different times on the average over the two seasons, and must therefore carry alleles with different additive effects (since non-allelic interaction is apparently not involved). The flowering order of the parents was 7 8 5 6 2 4 1 3 in 1951 and 7 8 5 2 4 1 6 3 in 1953. Only one shift was significant, namely, that of parent 6 relative to all other parents. It may therefore be concluded that the additive effects of the genes of all the parents except No. 6 were stable over the two seasons.

Turning to the analysis of the variances and covariances of the arrays of the diallel table, it is apparent from the Years item in table 4 that there was a real difference between $\bar{W}_r + \bar{V}_r$ in the two years. Geometrically, this indicates that the point representing the mean for 1953 lies on the regression line at a position farther from the origin than the comparable point for 1951. As we shall see below, mean dominance was similar for the two years. Hence, the significant variance ratio for years probably resulted from failure of the rescaling to equalize completely the mean additive effects of the genes governing flowering time.

The significance of the Dominance item shows that $D \neq H_1$ i.e., that mean dominance over the two years was not complete. (As we have seen, the regression lines for 1951 and 1953 each suggest partial dominance). The non-significance of the Years × Dominance item shows that the regression lines representing the two years occupy the same positions, or in other words, that the ratio of H_1/D was consistent in 1951 and 1953.

TABLE 4

Analysis of variance of W_r *and* V_r *for flowering time for 1951 and 1953*

Component of variation	d.f.	Sums of squares	Variance ratio	Probability
Years	1	0.2265	119.21	<0.001
Dominance	1	0.1846	97.16	<0.001
Years × Dominance	7	0.0001		
Arrays	7	2.8856	216.95	<0.001
Years × Arrays	7	0.7446	55.95	<0.001
Dominance × Arrays	7	0.0209	1.53	>0.20
Years × Dominance × Arrays	7	0.0130	0.95	>0.20
Error	32	0.0631		

Finally, the significance of the Arrays component of variation indicates that certain of the parents did differ from one another in dominance. In fact, 6 of the 8 parents differed significantly from each other ($P < 0.01$). Furthermore, the significance of the Years × Arrays item shows that the points representing the 8 parents moved relative to one another up or down the regression line, i.e., that the parents did not maintain the same dominance relationships. In 1951 the dominance order was 8 5 2 6 7 4 1 3, but in 1953 it had become 7 5 4 2 8 1 6 3. Of the 28 comparisons possible, all except 7 were significant ($P < 0.01$). The most stable arrays were the highly dominant ones, Nos. 2, 5, and 8, and the most recessive, No. 3. The most unstable array was No. 6, varying as it did from one of the more dominant in 1951 to recessiveness in 1953. Arrays 1, 4, and 7 were moderately unstable in their dominance relationships. The method is clearly quite sensitive in detecting changes in dominance order.

The results with the flowering-time data thus suggest that the additive effects of genes, as measured by analysis of the parental means, were quite stable in the two seasons. Dominance relationships among various parents were much affected by the play of environment. But the shifts in dominance that occurred among the individual parents balanced one another, since they did not upset the mean dominance relationships over all arrays. The non-significance of the Dominance × Arrays item provides additional evidence that non-allelic interaction is unimportant in the inheritance of flowering time.

ANALYSIS IN THE PRESENCE OF NON-ALLELIC INTERACTION

The form of the (W_r, V_r) graphs for flowering time suggests that the assumptions, enumerated above, upon which the diallel analysis is based, were fulfilled for that character in 1951 and 1953. Under those circumstances the method illustrated was capable of detecting a variety of genetic-environmental interactions. The method detects non-allelic interaction and other disturbances when they are a sporadic feature of the genetic system, and it provides procedures whereby the offending hybrids can be eliminated and the remainder analyzed. This raises the question of the interpretation of genetic-environmental interactions when epistasis is a regular feature of the genetic system. Obviously, a model based upon d and h will no longer

be adequate, but additional parameters representing interactions between loci will be required.

The development by several workers (COCKERHAM 1954; ANDERSON and KEMPTHORNE 1954; HAYMAN and MATHER 1955) of a system of notation permitting non-allelic interactions to be described in a general way provides a method of approach to this problem. In HAYMAN AND MATHER'S notation the genotypes *AABB*, *AAbb*, *aaBB*, and *aabb* have the following phenotypes:

$$\begin{array}{ll} AABB & d_a + d_b + i_{ab/} - \tfrac{1}{2}j_{a/b} - \tfrac{1}{2}j_{b/a} + \tfrac{1}{4}l_{/ab} \\ AAbb & d_a - d_b - i_{ab/} - \tfrac{1}{2}j_{a/b} + \tfrac{1}{2}j_{b/a} + \tfrac{1}{4}l_{/ab} \\ aaBB & -d_a + d_b - i_{ab/} + \tfrac{1}{2}j_{a/b} - \tfrac{1}{2}j_{b/a} + \tfrac{1}{4}l_{/ab} \\ aabb & -d_a - d_b + i_{ab/} + \tfrac{1}{2}j_{a/b} + \tfrac{1}{2}j_{b/a} + \tfrac{1}{4}l_{/ab} \end{array}$$

where d_a and d_b denote the additive effects of genes *a* and *b*, $i_{ab/}$ the interaction between d_a and d_b, $j_{a/b}$ and $j_{b/a}$ the interactions between d_a and h_b and d_b and h_a respectively, and $l_{/ab}$ the interaction between h_a and h_b. With all classical types of interaction $i_{ab/}$, $j_{a/b}$, $j_{b/a}$ and $l_{/ab}$ are equal. Using i to denote the several types of interaction, the variances and covariances of a diallel of the four genotypes given above are shown in table 5.

Generalization over many genes does not affect the additive (d) or dominance (h) components of variation in any fundamental way, but the interaction components (terms involving i) become very complex in multigenic models. A full investigation of diallel analysis of multigenic models has not been made. It is of interest, however, that the classical types of interaction give the same pattern of results for 3 and 4 gene models as with the digenic model.

The increased complexity of W_r and V_r caused by epistasis makes the genetic interpretation of the components of variation of the analysis of variance table considerably more difficult. Thus, the Years component of variation is now influenced not only by changes in dominance but also by changes in non-allelic interactions, assuming that rescaling has eliminated inter-seasonal differences in the additive effects of genes (see table 5). Non-significance of the Years item can be taken as evidence that H_1, F, and non-allelic interactions are stable and the rescaling was effective. Significance indicates that one or more of these postulates is incorrect.

TABLE 5

Covariances and variances of arrays of a two-gene, four-line diallel experiment where $i_{ab/} = j_{a/b} = j_{b/a} = l_{/ab}$, *denoted by* i, *and* u = v

	W_r	V_r
AABB	$2d_a^2 + 2d_b^2 - 2d_ah_a - 2d_bh_b - d_ai - d_bi + h_ai + h_bi$	$d_a^2 + d_b^2 + h_a^2 + h_b^2 - 2d_ah_a - 2d_bh_b$
AAbb	$2d_a^2 + 2d_b^2 - i^2 - 2d_ah_a + 2d_bh_b - d_ai + d_bi + h_ai - h_bi$	$d_a^2 + d_b^2 + h_a^2 + h_b^2 + i^2 - 2d_ah_a + 2d_bh_b + 2d_bi + 2h_bi$
aaBB	$2d_a^2 + 2d_b^2 - i^2 + 2d_ah_a - 2d_bh_b + d_ai - d_bi - h_ai + h_bi$	$d_a^2 + d_b^2 + h_a^2 + h_b^2 + i^2 + 2d_ah_a - 2d_bh_b + 2d_ai + 2h_ai$
aabb	$2d_a^2 + 2d_b^2 + 6i^2 + 2d_ah_a + 2d_bh_b - 3d_ai - 3d_bi - h_ai - h_bi$	$d_a^2 + d_b^2 + h_a^2 + h_b^2 + 6i^2 + 2d_ah_a + 2d_bh_b - 2d_ai - 2d_bi - 2h_ai - 2h_bi$

The Dominance component of variation is no longer concerned exclusively with mean dominance but is also influenced by non-allelic interactions (table 5). Its significance is evidence either that dominance is not complete, or that epistasis is involved, or both. The Years × Dominance item tests the constancy of mean dominance effects and/or epistatic effects in different seasons, non-significance suggesting stability.

The Arrays component of variation can be non-significant only if all dominance and epistatic effects of the genes are zero (table 5). Significance of this component is indicative of either significant dominance and/or non-allelic interactions. The Years × Arrays item tests the stability of mean dominance and non-allelic interaction effects for each parent over seasons, non-significance again indicating stability.

The Dominance × Arrays component of variation is independent of fluctuations in the additive and dominance effects of genes with classical types of interaction systems (table 5). Since differences in $W_r - V_r$ for arrays can occur only if $i \neq 0$ in such systems, significance of the Dominance × Arrays component provides evidence that epistasis is a feature of the genetic system. The Years × Dominance × Arrays item clearly tests whether the expression of the non-allelic interaction is the same in different environments.

In a two-gene four-parent model with $d_a = d_b$ and $h_a = h_b$, the sums of squares for arrays is

$$8d^2h^2 + 6d^2i^2 - 8d^2hi + 20dhi^2 - 12di^3 + 3h^2i^2 - 9hi^3 + \frac{27}{4}i^4$$

and the Dominance × Arrays sums of squares is

$$h^2i^2 + hi^3 + \frac{1}{4}i^4.$$

If $i = 0$, it follows that the ratio of the mean square of arrays to the mean square of Dominance × Arrays will not differ significantly from unity provided that h also is zero. The ratio will, however, increase in magnitude as dominance increases. If $h = d$ and i has a positive value, as with recessive epistasis or complementary gene action (HAYMAN and MATHER 1955), the variance ratio will be small. If i has a negative value—as with duplicate type gene action, dominant epistasis, or gene action of the recessive suppressor type—the ratio will be very large. It is, of course, possible to calculate expected values for this ratio for types of non-allelic gene action other than the classical types considered above. Over-duplicate and under-complementary types, for example, give very large and very small ratios, respectively.

NUMERICAL EXAMPLE

The analysis will be illustrated using height data taken from the same plants as the flowering time data. The (W_r, V_r) graphs for height for 1951, 1952, and 1953 are given in figure 2, from which it will be noted that complementary type of gene action appears to be a constant feature of the inheritance of height. As a first step in the interpretation of the variability, an analysis of variance was performed on the

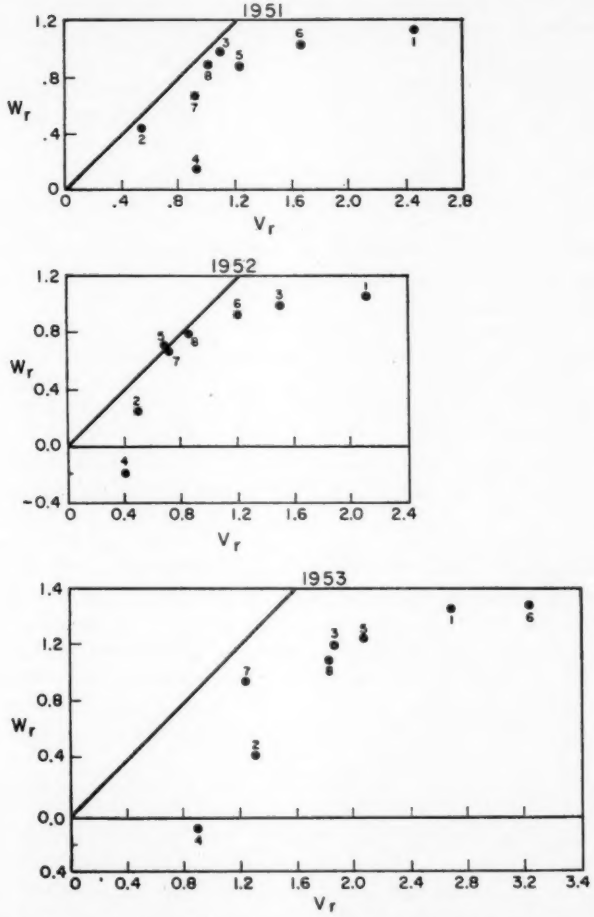

FIGURE 2.—(W_r, V_r) graphs for height for 1951, 1952, and 1953. Points represent the positions of parental arrays in relation to the regression line of unit slope through the origin. See text for interpretation.

heights of the parents (table 6). The Years item was significant, and, in fact, the mean height was different for each of the three years. It can, therefore, be concluded that the absolute values of the additive effects and/or the interactions between homozygous loci (interactions of the $i_{/ab}$ type) altered with environmental changes. The 8 parents formed 4 groups with respect to mean height over the three years, an indication of diversity with respect to the d and/or $i_{/ab}$ effects of the genes of several parents. The Parents × Years item was also significant. In fact, the only parents

TABLE 6

Analysis of variance of the mean heights for eight parental varieties in 1951, 1952, and 1953

Component of variation	d.f.	Sums of squares	Variance ratio	Probability
Years	2	6,168.75	224.31	<0.001
Parents	7	7,620.99	79.17	<0.001
Years × Parents	14	1,150.11	5.97	<0.001
Error	24	330.10		

that behaved consistently relative to one another were 1–3, 2–4, 3–8, 4–5, 4–8, 5–6, and 5–8. This provides evidence that d and/or $i_{/ab}$ type interactions were quite labile in response to environment.

The analysis of variance of W_r and V_r for the three years is given in table 7. From the significance of the Years item it can be seen that a real difference existed between $\bar{W}_r + \bar{V}_r$ over the three years. This might be interpreted as indicating that mean dominance or epistatic effects altered in each year, although other causes are possible as we have seen earlier.

The Dominance item is also highly significant. This would be expected if the degree of dominance is other than complete and/or non-allelic interaction is involved. The regression line has a slope of less than unity. That this is due to epistasis is supported by the significance of the variance ratio for the Dominance × Arrays component of variation.

The Years × Dominance item provides a measure of the stability of the mean effects of dominance and non-allelic interaction over years, its variance ratio in the present instance suggesting moderate instability. It should be noted that the Years × Dominance × Arrays item is non-significant, an indication that epistatic effects were stable over years. By thus eliminating instability of epistatic effects, shifts in mean dominance effects seem to be identified as responsible for the significance of the Years × Dominance component of variation.

The Arrays component is significant, and, in fact, comparisons between the 8 arrays showed them all to differ significantly from one another. The 8 points representing the mean values for the arrays over the three years thus occupied different positions on the regression line, i.e., were scattered along the regression line at intervals sufficiently great to be significant. It may therefore be concluded that the 8 parents are all unique with respect to the dominance and/or epistatic effects of the genes they carry.

The stability of the dominance and epistatic effects of genes of the individual parents is tested by the Years × Arrays component of variation. It is not significant, which can be interpreted geometrically as indicating that the points for arrays retained their positions relative to one another along the regression line. The genetical interpretation is one of stability of the h and i effects (recalling that i denotes general interaction) of the genes of the parents in the three environments. Thus, although changes in h and i among individual parents were not great enough to result in significance of the Years × Arrays or Years × Dominance × Arrays items, the fact that h and/or i were not completely stable is attested by the sig-

TABLE 7

Analysis of variance of W_r *and* V_r *for height for 1951, 1952, and 1953*

Component of variation	d.f.	Sums of squares	Variance ratio	Probability
Years	2	5.4498	25.87	<0.001
Dominance	1	6.7109	63.78	<0.001
Years × Dominance	2	0.9504	4.52	0.01–0.001
Arrays	7	20.6132	27.95	<0.001
Years × Arrays	14	2.4700	1.67	>0.20
Dominance × Arrays	7	2.7402	3.72	0.01–0.001
Years × Dominance × Arrays	14	0.7796	0.53	>0.20
Error	48	5.0525		

nificance of their summed effects, as measured by the Years × Dominance item. The non-significance of the Years × Dominance × Arrays item provides direct evidence that i was stable over years. The combined evidence suggests, therefore, that small changes must have occurred in h and, furthermore, that they were non-compensating or unidirectional changes such that their summed effects over all arrays were large enough to result in significance of the Years × Dominance component of variation. It can be seen from table 7 that the components of variation that are sensitive to changes in dominance effects all had small variance ratios, thereby suggesting comparative stability of dominance in the three environments under consideration, even though the variance ratios sometimes were large enough to be significant.

It has already been noted that the Dominance × Arrays and Years × Dominance × Arrays items show non-allelic interaction to be involved in the inheritance of height and that the pattern of the epistasis was nearly identical in the three seasons. The (W_r, V_r) graph suggests that the type of non-allelic interaction involved was complementary in nature. The ratio of the mean squares of Arrays and Dominance × Arrays was 7.52, a ratio of the order of magnitude expected if i had a positive value. Among the classical types of interaction, positive values of i occur with recessive epistasis or complementary type of gene action. This analysis therefore leads to the same conclusion as the (W_r, V_r) graph.

Re-examination of the flowering-time data for 1951 and 1953, where a genetic system featuring partial dominance and no epistasis was postulated from the (W_r, V_r) graph, proved to be illuminating. In those two years the Arrays/Dominance × Arrays mean square ratios were 68.0 and 141.9, ratios of the general magnitude expected with partial dominance.

SUMMARY

In the genetical description of continuous variation, the phenotypic effects of genes can be specified in many cases by three types of parameters, d, h, and i, denoting the increments due to additive effects of genes, dominance, and non-allelic interactions, respectively. An extension of the analysis of diallel crosses is presented which permits an assessment of the stability of the values assumed by d, h, and i under different environmental circumstances.

Application of the method to date of flowering and height data for intervarietal hybrids of *Nicotiana rustica* suggested that non-allelic interaction was unimportant in the genetic control of the former but a regular feature of the genetic control of the latter character. For date of flowering the additive genetic effects were comparatively stable, but the dominance effects of genes appeared to be quite unstable in different environments. In the genetic control of height non-allelic interaction of a complementary type was inferred, and it proved to be quite stable in different environments. Dominance relationships in the genetic control of height appeared to shift only moderately with environmental changes, but the additive effects of genes controlling height were altered drastically by environmental changes.

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TESTS FOR NON-ALLELISM AT THE PYRIMIDINE-3 LOCUS OF NEUROSPORA

MARY B. MITCHELL AND HERSCHEL K. MITCHELL

Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California

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AMONG the pyrimidine-requiring mutants of Neurospora is one which exhibits the requirement only at temperatures above 31°, in contrast to the more frequently isolated mutants which show an absolute requirement regardless of the temperature of incubation (MITCHELL and HOULAHAN 1947). One of the latter mutants was found to be allelic or quite closely linked to the temperature-sensitive one and to be suppressed by a suppressor of this mutant (HOULAHAN and MITCHELL 1947). More recently a third mutant has been found to be closely linked or allelic to these but is apparently unaffected by the suppressor. This mutant has been shown to give pseudo-wild progeny when crossed to either of the other two (MITCHELL, PITTENGER and MITCHELL 1952). The study which is described here was undertaken in an attempt to learn whether the three mutants are non-allelic.

RESPONSE TO THE SUPPRESSOR

The three pyrimidine mutants examined are *pyr-3a* (37301) and *pyr-3d* (45502), not temperature-sensitive, and *pyr-3b* (37815), temperature-sensitive. They will be referred to hereafter as *a*, *b* and *d*. All isolates of *d* used are derived from one which, according to genetic evidence (MITCHELL, PITTENGER and MITCHELL 1952), has been freed of the chromosome rearrangement found in the original isolate of this mutant (MCCLINTOCK 1945). The "colonial" mutant, *co* (70007) has been used as a marker. It is located about 10 to 13 units from the centromere in linkage group IV and about two to three units nearer the centromere than *pyr-3* (MITCHELL and MITCHELL 1954).

Evidence that mutants *a* and *b* respond to the suppressor has been presented (HOULAHAN and MITCHELL 1947). In order to show that *d* recombines with the suppressor gene and is not affected by it, use was made of an arginine mutant (33442) which responds to the suppressor but in a negative way. This mutant, which is located between *co* and *pyr-3*, shows an absolute requirement for arginine if the suppressor gene is present but grows slowly on unsupplemented medium when this gene is replaced by its normal allele (MITCHELL and MITCHELL 1952). Spore pairs of asci from the cross, *arg s* × *co pyr-3d*, were isolated and tested. None of these showed recombination with respect to *co*, *arg*, and *pyr*. Tetrads, of which one or both of the *arg* isolates were found not to carry *s*, were further tested by obtaining dry weights of mycelium produced on suitably supplemented media. The *pyr* isolates which presumably carried *s* were not found to differ in any way from those which must have carried its normal allele.

HETEROCARYONS FROM PSEUDO-WILDS

MITCHELL, PITTENGER and MITCHELL (1952) have shown that pseudo-wilds, isolated from crosses of *co d* × *a*, behave in outcrosses as if they are heterocaryons composed of *co d* + *a*. More recently pseudo-wilds from crosses of *co d* × *b* have been isolated in the same way, that is, by selecting strains which arose from single, normal sized ascospores and which were phenotypically wild with respect to *pyr* on minimal agar plates. The cross, *co b* × *a*, has been found to produce strains which correspond to pseudo-wilds in every way except that their growth on minimal medium is slow and erratic. They are, however, clearly less pyrimidine-dependent than either of the parent mutants.

When pseudo-wilds involving these mutants were first observed it was not known that pyrimidine-independent heterocaryons involving them could be obtained. Attempts to obtain such heterocaryons by mixing different ascospore isolates of the mutants had been consistently unsuccessful. For this and other reasons the possibility was considered that the pseudo-wilds were derived from spores whose nuclei were disomic with respect to the chromosome carrying the *pyr* linkage group and that the pyrimidine-independence of the pseudo-wilds was due to the persistence of at least some nuclei in this form (MITCHELL, PITTENGER and MITCHELL 1952). It was clear, however, that many fully haploid nuclei were present and PITTENGER (1954) was able to show that the pure parent mutants could be recovered from some of the conidia produced by a pseudo-wild. Attempts were, therefore, made to reconstitute the pyrimidine-independent pseudo-wilds by mixing mutant strains obtained in this way, from the same pseudo-wild. Conidia from one of each of the three types of pseudo-wilds described above were plated on minimal medium and incubated at 34°C for about 17 hours. Heterocaryotic conidia germinate and begin to grow, whereas conidia, which are pure with respect to any one of the *pyr* mutants, swell so that they can be seen and isolated under a dissecting microscope but they do not produce germ tubes, except for *b* conidia, some of which produce very short ones. Mutant conidial isolates, established on complete medium slants, were tested and found to be like the parent mutants. On minimal slants, mixed cultures were made of the pairs of mutants from one pseudo-wild from each of the three crosses, *co b* × *a*, *co d* × a and *co d* × *b*, in order to see whether they were capable of forming pyrimidine-independent heterocaryons. Growth from these mixtures was like that of the pseudo-wilds themselves. The mixture *co b* + *a* grew slowly but produced a fully grown culture at 34°C. The other two grew as rapidly as wild. All appeared to be mixed with respect to *co* and its normal allele, as do the pseudo-wilds. This test was repeated several times and each time growth of the mixture duplicated that of the pseudo-wild from which it was derived. From *co d* + *b*, "hyphal tip" isolates, or fragments of mycelium which could be seen to have come from single strands, gave, when tested in flasks of minimal medium, dry weights like those from the corresponding pseudo-wild. From these results it seems clear that the pyrimidine-independence of the pseudo-wilds is not due to the persistence of disomic nuclei.

GENETICALLY-WILD PROGENY FROM INTERCROSSES

Several years ago the crosses, *a* × *d* and *b* × *d*, were observed to give occasional genetically-wild spores as well as pseudo-wilds, but attempts to learn whether these

arise from crossing over between the *pyr* mutants have not given satisfactory results for several reasons. The two crosses are almost sterile; it has been estimated that not more than 5% of the spores formed are capable of germinating and no complete asci have been found. Hence it is not possible to learn whether the true-wild progeny are accompanied, in the same ascus, by the double mutant. Presumably it would not even be possible to detect the double mutant among random spores since *ad* would not be expected to differ phenotypically from either parent and *bd* would be expected to be phenotypically like *d*. A nearby marker distal to *pyr* is not known at present. However, studies of random spores from intercrosses in which *co* was heterozygous have shown that the wild progeny cannot all have resulted from single crossovers in the region, *co* to *pyr*, (about three units) since, from the same cross, *co* + was found nearly as frequently as ++.

The counts given in table 1A were obtained from crosses involving several different isolates of each strain. One isolate of each of the three *pyr* mutants was then crossed to the same isolate of *co* and from the progeny of these crosses, one isolate of each strain was selected for use in the crosses listed in table 1B. With one exception, given as an approximate number, the figures for the numbers of *pyr* mutant spores in table 1A are actual counts. In table 1B they are estimates, but, in both cases, the plates were examined under the microscope to detect spores which were wild with respect to *pyr*. The strains classified as + + could be distinguished from pseudo-wilds on the plates because of the characteristic phenotype which the pseudo-wilds maintain during the first 24 hours growth from an ascospore. They could also be distinguished from strains derived from two other types of spores which give rise to heterocaryotic mycelia and which have been found from these and other

TABLE 1

Wild "recombinants" from intercrosses

	Spore types		
	pyr	++	*co* +
A-cross			
co b × *a*	28318	0	0
	28000 (approx.)	0	0
co d × *a*	17080	9	4
co d × *b*	26782	1	1
b × *a*	23888	0	—
b × *b*	19277	0	—
B-cross			
co b × *a*	18000	0	0
co d × *a*	15000	10	8
co d × *b*	25500	6	2
co b × *b*	20000	0	0

In the A group, results from crosses involving several different isolates of each strain are lumped together and, except for the figure marked "approximate," the numbers are actual counts. All crosses of group B involve only one isolate of each strain and the number of *pyr* mutant progeny was estimated.

crosses. In the case of one of these the length of the spore is double or more the normal length and, from other crosses, such spores have been found in asci containing less than eight spores (MITCHELL 1954). Spores of the other type are also larger than normal, often twice as large, but normal in shape. From other crosses they have been found in asci containing eight such spores. The origin of these asci is not known. On agar plates the mycelia of both types, from the crosses under consideration, differ from those produced by true-wilds and pseudo-wilds in several ways. They usually grow more slowly and appear impure with respect to the *co* character, some branches showing this character and some not. When isolated they were, as often as not, found to be heterocaryotic with respect to mating type as well as to the mutants of the *pyr* linkage group. There is, of course, no difficulty in distinguishing *co* + from any of the other types mentioned since the pure *co* phenotype is quite distinct.

The following isolates, classified on plates, have been outcrossed and found to be genetically wild with respect to *pyr*: from *co d* × *a*, four ++ and one *co* +; from *co d* × *b*, two ++ and one *co* +. From *co d* × *a*, three ++ (including one of those above) and one *co* + were crossed to one or the other of the parent mutants. These crosses were fertile and gave 1:1 segregation of *pyr* and + in asci.

STERILITY IN INTERCROSSES

A characteristic of the *pyr* mutants, which has been as consistently observed as has their requirement for pyrimidine, is the sterility and partial sterility of certain crosses involving them. The sterile crosses produce, in asci, spores which remain colorless, or nearly so, and do not germinate. From the partly-sterile crosses by far the greater proportion of spores are of this type, but there are some which darken normally and are capable of germinating. There seems to be no association of normal spores in pairs in asci, and asci with eight normal spores have not been found. Crosses classified as fertile vary somewhat in fertility, depending on the isolates used, as do even wild × wild crosses, but many asci containing eight normal spores are produced.

Table 2 gives a classification of crosses involving *a*, *b*, and *d* and also several other *pyr* mutants which have not been shown to be at different loci. Although these other mutants have not been as extensively studied as have *a*, *b* and *d*, they are included here because they seem to follow the same pattern of behavior. Mutant *f* (49001) is of special interest because it has behaved like *d* in every way except that there is no indication that it carries the chromosome rearrangement which was present in the original isolate of *d*. Like *d*, it fails to respond to the suppressor of *a*, *b* and *c*. Mutants *e* and *g* have not been tested with the suppressor. They are considered to be possible recurrences of *a* since they have behaved like it in all tests performed. Mutant *c* is much like *b* except that it shows greater pyrimidine dependence at 25°C (MITCHELL and HOULAHAN 1947). Six other mutants (44504, 47102, 63902, 63904, 68305 and 68902), which are phenotypically like *a* or *d*, have been repeatedly observed to give sterile or almost sterile crosses to *a* or *d*, although they are fertile in crosses to wild.

BEADLE and COONRADT (1944) have reported a method, later used successfully in a modified form by LEIN and LEIN (1952), for improving the fertility of crosses by first establishing a heterocaryon composed of one of the desired parents and an unrelated mutant and crossing this heterocaryon to the other parent. This was tried

TABLE 2

Classification of crosses with respect to fertility

Fertile	Almost sterile	Sterile
a (37301) × +	*a* × *d*	*a* × *a*
b (37815) × +	*a* × *f*	*a* × *e*
c (67602) × +	*b* × *d*	*a* × *g*
d (45502) × +	*c* × *d*	*d* × *d*
e (1298) × +	*d* × *e*	*d* × *f*
f (49001) × +	*d* × *g*	
g (44101) × +		
a × *b*		
a × *c*		
b × *b*		
b × *c*		
b × *e*		

in connection with crosses of *a* and *d* to several mutants of the same phenotype. No difficulty was encountered in obtaining heterocaryons of *pyr* with several unrelated mutants requiring proline, pantothenic acid, or isoleucine plus valine. However, if any effect was produced on the sterility of the *pyr* × *pyr* cross it was not detected, since, in all cases, all progeny obtained from asci of crosses of the heterocaryons to *pyr* were derived from the unwanted cross, that is, of the unrelated mutant × *pyr*. No effect whatever was observed to result from changes in the medium on which the crosses were made (increase or decrease in pyrimidine concentration, different pyrimidines as supplement, different basal media, etc.) or from lowering the temperature of incubation from 25°C. Also, suppressed *a* exhibits the same sterility as *a* unsuppressed.

It is striking indeed that any one of the mutants is fertile when crossed to wild whereas the only fertile *pyr-3* × *pyr-3* crosses involve either *b* or *c*, in which the mutant character is not fully expressed at temperatures which allow the development of crosses. It is known, moreover, that not all crosses between not temperature-sensitive *pyr* mutants are sterile, from the fact that mutants which have been shown to be genetically different (by the isolation of both wild type and the double mutant from the same ascus) give fertile crosses. For example, crosses of *pyr-3a* and *d* to *pyr-1* (263) and *pyr-4* (36601) are fertile. (It might be mentioned here that *pyr-4* is not likely to be an allele of 44302, as BARRATT, NEWMEYER, PERKINS and GARNJOBST (1954) have suggested. Mutant *pyr-4* does not show linkage to *pyr-1*, *pyr-2* (38502) or to *pyr-3a*, whereas 44302 shows rather close linkage to *pyr-1* and has not been observed to recombine with members of the *pyr-3* group. It seems more likely that 44302 is a recurrence of *pyr-3c*, from which it is phenotypically indistinguishable.) In considering mutants *a* and *d* it may be said that when the wild type allele is known to be present the cross is fertile, but when the wild allele is known to be absent, as in *a* × *a* and *d* × *d*, the cross is sterile. Since the cross, *a* × *d* is almost sterile one might conclude that the wild allele of neither mutant is present. In other words, *a* and *d* represent different mutations at the same locus and the

heterozygote involving them is mutant. From this point of view *b* and *d* would also appear to be allelic and to form a heterozygote in which *d* is dominant.

The observations that the fertility of *pyr* × *pyr* was not, apparently, increased in crosses of the heterocaryons, *pyr* + unrelated mutant × *pyr*, suggests that the wild allele of *pyr* must be in the developing ascus in order to produce an effect. Therefore, although it is true that the crosses *a* + *d* × *a* and *a* + *d* × *d*, are no more fertile than *a* × *d*, this gives no further information regarding the question of allelism here than that provided by the cross *a* × *d*.

No progeny that are wild with respect to the pyrimidine requirement have been obtained from the fertile intercrosses, whereas they have been found from all crosses listed as almost sterile in table 2. It will be noted that the latter crosses all involve *d* or *f*, which is, quite possibly, a recurrence of *d*.

From these crosses all *co* + and ++ isolates which have been tested have given fertile crosses to wild, to one another and to either of the parent mutants. Whatever the mechanism through which these "recombinants" arise, it is able to dispose of both the requirement and the sterility, whereas in the heterocaryons and in suppressed *a* only the requirement is lost. The sterility remains.

DISCUSSION

In attempting to evaluate the foregoing information in connection with the question of whether the *pyr* mutants are non-allelic, it would appear that reactions with the suppressor may be disposed of rather briefly. These reactions simply give no information bearing on the question. It was, at one time, suggested (HOULAHAN and MITCHELL 1947) that suppression of *pyr-3a*, *b*, and *c* by *s* and failure of *pyr-1* and *-2* to be suppressed indicated that *3a*, *b*, and *c* are alleles. The naivete of this suggestion later became apparent when it was found that *s* also suppresses two non-allelic arginine-requiring mutants (MITCHELL and MITCHELL 1952). However, failure of *pyr-3d* to be suppressed is surely indicative of some sort of difference between it and *a* and *b*.

The heterocaryon test for non-allelism, based on the assumption that allelic mutants cannot show a loss of the growth-factor requirement in a heterocaryon, has been extremely useful in screening large numbers of similar mutants for ones which are possibly different genetically. And, when duplicate mutations are being considered, the assumption seems an obviously safe one. However, to the authors, at least, it does not seem possible to predict the reactions, in heterocaryons, of members of a series of multiple alleles. It seems possible that some combinations may be capable of interaction which would remove the requirement, depending upon the nature of the effects of the mutations on the physiological processes, and upon the extent of difference in the effects of the two mutations. For this reason the fact that the combinations, *a* + *d* and *b* + *d*, are capable of forming pyrimidine-independent heterocaryons is not considered to constitute acceptable evidence of non-allelism. In the case of *a* + *b*, the fact that the heterocaryon is only partly independent might be considered to provide an argument against non-allelism. If the two mutations introduce partially overlapping defects, then, presumably, no interaction between them could completely remove the requirement. However, it may also be

imagined that the genetic background necessary for complete independence is, in this case, simply not available in the isolates used (GARNJOBST 1953; HOLLOWAY 1955).

From the sterility of the intercrosses one might conclude, simply, that *a*, *b*, and *d* are allelic, since the heterozygotes *a*/*d* and *b*/*d* are mutant. However, in the classic cases of pseudoallelism in Drosophila (GREEN 1955; LEWIS 1955) it has been demonstrated that, although the trans heterozygotes are mutant, the mutant genes are, nevertheless, separable by crossing over.

A demonstration that the pyrimidine mutants are separable by crossing over would, in the authors' opinion, consist in showing that in the intercrosses the double-mutant recombinant and the wild type are formed at the same meiosis, i.e., that these two recombinants occur in equal numbers in the same ascus. This has been the routine procedure used in Neurospora work to show that two mutant genes occupy different loci. Since complete tetrads from the *pyr* intercrosses cannot be analyzed it appears unsafe to assume that wild types found among random spores are due to crossing over between the *pyr* mutants, particularly since the distribution of the marker, *co*, is not as expected from single crossovers. That this assumption is also unnecessary has been emphasized by the results of a study of some pyridoxine mutants (MITCHELL 1955a, 1955b) whose behavior is similar in several aspects to that of the pyrimidine mutants. Analysis of tetrads from the pyridoxine intercrosses showed that wild segregants occurred which were not accompanied in the same tetrad by the double mutant. The double mutant has not, in fact, been shown to exist. The behavior of the pyridoxine mutants strongly suggests that wild types from the intercrosses are due to the phenomenon encountered by DEMEREC (1928) in a study of mutants of *Drosophila virilis* and described as reverse mutation associated with crossing over in heterozygotes. Wild individuals from the pyrimidine intercrosses could also be explained in these terms. One need only suppose that in the crosses, $a \times d$ and $b \times d$, mutant *d* sometimes undergoes reverse mutation when there is a crossover nearby, either to the right or to the left. Therefore, it is not assumed, in the absence of the required evidence, that these mutant genes are separable by crossing over. The possibility exists that they are so separable but in the authors' opinion all aspects of their behavior so far observed can be accounted for in terms of multiple allelism (WRIGHT 1925).

SUMMARY

Of three mutants examined, *a* and *d* are phenotypically alike and differ from *b* in that the latter shows its mutant character only at higher temperatures. A suppressor acts on both *a* and *b* but fails to suppress *d*. In heterocaryons, the combinations $a + d$ and $b + d$, are pyrimidine-independent, whereas the combination, $a + b$, although it is less dependent than either component, shows a partial requirement for pyrimidine. No wild progeny have been observed from the cross, $a \times b$, but both $a \times d$ and $b \times d$ give occasional wild offspring. It has not been demonstrated that the wild types arise as a result of crossing over between the pyrimidine mutants. Although *a* and *d* are fertile in outcrosses, each is completely sterile when crossed to itself and the cross, $a \times d$, is almost sterile. Mutant *b* is fertile in outcrosses, crosses

to itself and to *a*, but it is almost sterile when crossed to *d*. Since the three mutants have not been shown to be separable by crossing over it is not assumed that they are non-allelic or pseudoallelic. Their behavior is considered to be consistent with the possibility that they represent different mutations at the same locus.

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MUTATIONS OF SELF-INCOMPATIBILITY ALLELES IN TRIFOLIUM PRATENSE AND T. REPENS

K. K. PANDEY

John Innes Horticultural Institution, Bayfordbury, Hertford, Herts, England

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THE existence of several allelomorphs at the *S* locus has been demonstrated in Nicotiana spp. (EAST and YARNELL 1929), *Veronica syriaca* (LEHMANN 1926), *Antirrhinum glutinosum* (GRUBER 1932), *Trifolium pratense* (WILLIAMS 1939; and WILLIAMS and WILLIAMS 1947), *Oenothera organensis* (EMERSON 1939, 1940) and *Trifolium repens* (ATWOOD 1944). In *Trifolium pratense*, BATEMAN (1947) calculated that the number of *S* alleles operating in the material investigated was 212.

The unusually high number of *S* alleles may have arisen in any of the following ways: (1) Direct mutation of one allele into another—any new mutation will have a selective advantage over the existing alleles and will easily spread into the population. It is expected that such new 'constructive' mutations, if they do occur in nature, could also be artificially induced. (2) An allele may give rise to a new allele in several steps, due to some balanced changes in its internal structure. The small initial selective advantage may ultimately lead to a new fully self-incompatible *S* allele. (3) All the *S* alleles may have been created independently from the original *S* gene complex (by the (2) method) during the course of evolution of the incompatibility system. In this case no mutation of one allele into another is involved. The technique of investigation may not be sensitive enough to pick up such minute changes as may be occurring in (2) and (3).

A study of the spontaneous and X-ray induced mutations in the *S* gene in *Prunus avium* and *Oenothera organensis* by LEWIS (1948, 1949 and 1951) showed no indication of 'constructive' mutation. It was found that the *S* gene was composed of two parts, the part governing the reaction of pollen, and the part governing the reaction of style. Mutation could take place in one or both of these parts. But these changes always brought about self-compatibility and no evidence was found for the creation of a new *S* allele from another by direct mutation.

The present study of spontaneous and X-ray induced mutations in *S* alleles of *Trifolium pratense* and *Trifolium repens* is directed towards further information regarding the nature of the *S* allele and its bearing upon the high number of these alleles encountered in the natural population.

MATERIAL

Seeds of unrelated crosses in *T. pratense* L. (2n = 14) and *T. repens* L. (2n = 32) were obtained from the Plant Breeding Station, Aberystwyth. Two families in *T. pratense* gave four intra-sterile inter-fertile groups. When one of these groups was crossed as a female parent with the other three groups of the same family, two of the three families in the next generation gave two groups each, and the third family gave four groups. By backcrossing these various groups with the parental groups, the geno-

type of each was established (ATWOOD 1940). Similarly *S* genotypes were established in other crosses of *T. pratense* and *T. repens*.

Seeds of *T. pratense* were also obtained from the Plant Breeding Station, Svalof, Sweden. A few plants raised from these seeds were used in the study of the rate of spontaneous and X-ray induced mutations.

TECHNIQUES

Induction of mutation

Plants were grown under normal glasshouse conditions, and flowered in May and June. For irradiation the pots were removed from the glasshouse to the X-ray room. Irradiation was done under uncontrolled room temperature. Soon after X-ray treatment plants were replaced in the glasshouse where they grew along with the control plants. Temperature and light conditions in the glasshouse were not controlled but the treated and untreated plants, for the present experiments, were kept, as far as possible, under identical conditions.

For spontaneous mutations, several hundred flowers were tripped (manipulation of the petals in order to simulate the visit of pollinating insects) for self-pollination. For inducing mutations by X-rays, very young inflorescences were chosen. It has been found that the incompatibility reaction is laid down in the cytoplasm at the time of tetrad formation, so that no mutation in the *S* allele which occurred after tetrad stage can express itself in the pollen (LEWIS 1949). This was also confirmed in pilot experiments in *T. pratense* and *T. repens*. For this reason the oldest bud in every inflorescence chosen for irradiation was tested for its stage of meiosis, and only those inflorescences which showed stages before tetrad were selected. Some of these were treated with different doses of X-rays (at 125 K.V.P., 5 M.A. and 15 cms distance) ranging from 750r to 3000r and others with a fixed dose of 750r which was found to be the best. When these inflorescences flowered they were tripped for selfing.

With this method pollen and egg are both irradiated. But there is no selection for mutations in the egg as they are unable to express themselves due to the interposition of diploid style between the egg and the pollen. Thus, whereas there is strong selection for the pollen reaction mutation in the *S* allele of the pollen grain, any mutation discovered in the *S* allele contributed by the egg will be purely accidental and must be rare.

Pollen estimation on stigma after tripping

In order to know the rate of mutation the proportion of the mutated pollen to the total number of pollen grains deposited on the stigma must be estimated. A mixture of four parts by volume of absolute alcohol to one part of chloroform has a density in which pollen grains of these species remain suspended. In 10 cc's of this mixture five stigmas from the tripped flowers (standard tripping—twice each flower, giving a thin, even layer of pollen grains on the stigma) were vigorously shaken for 10 minutes. 0.5 cc of such a suspension was poured into a counting dish divided with lines 1 mm apart and a drop of cotton blue was added. After the liquid evaporated counts were made under the microscope. Five such counts were made from each of the five or four bottles of 10 cc suspensions, prepared from each of the two species.

TABLE 1

Reactions of X plant when it is selfed, and crossed reciprocally with the parent. Different mutations generally give different reactions

Testing the mutant seedling

1	2		3		4		5	
TESTS	RT.		♂		♀		☿	
	HM.	HT.	HM.	HT.	HM.	HT.	HM.	HT.
X. SELFED	—	—	+	+	—	+	+	+
P. × X.	—	—	+	+	—	—	+	+
X. × P.	+	—	+	—	+	+	+	+

X. PLANT TESTED
P. PARENT
RT. REVERTIBLE
HM. HOMOZYGOUS
HT. HETEROZYGOUS

\+ COMPATIBLE
— INCOMPATIBLE

♂ POLLEN PART MUTATED
♀ STYLE " "
☿ BOTH PARTS "

Testing for mutation

All the plants derived from self-pollination were selfed and crossed reciprocally with the parent genotypes. Table 1 shows all the possible reactions after selfing and reciprocal crossing with the parents. In column 1, P and X stand for the parent plant and the plant obtained due to mutation, respectively. In column 2, RT. stands for 'Revertible' mutation (LEWIS 1951). These mutations are temporary and revert to their normal reaction before they are laid down into the seed. Plants obtained from such mutations show normal self-incompatible reaction. Some of these reactions are illustrated in table 2. The reactions in column 3 show that only the pollen (♂) reaction, and in column 4 only the style (♀) reaction, has changed, leaving the other part unaffected. In column 5 both pollen and style reactions (☿) have changed.

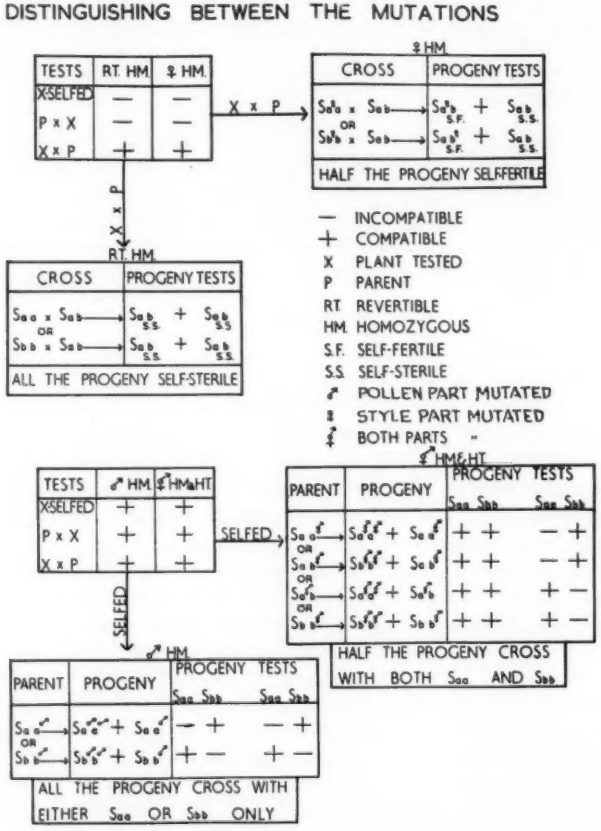
The normal plant usually being heterozygous for the *S* allele, the seeds obtained after selfing would give plants which would be either homozygous for one or the other allele, or heterozygous. The reactions for homozygous (HM.) and heterozygous (HT.) plants are different and have been given for each type of mutation.

The reactions in col. 5 (☿), when both pollen and style reactions have changed and those in the homozygous division of col. 3 (♂HM.), where only pollen reaction has changed, are the same and are thus indistinguishable without further test. These have been identified by testing the progeny obtained from the selfed seeds (table 2). This is done by crossing the progeny as the female parent with the two test plants, each of which is homozygous for one of the parental alleles. All the selfed progeny of the ♂HM. mutation will cross with only one of the test plants and will be incompatible with the other. In the case of ☿ mutation, half the selfed progeny will cross with both the test plants and the other half with only one.

Similarly, reactions of RT. HM. (col. 2) are indistinguishable from those of ♀ HM. (col. 4). Thus though a plant may be self-sterile it may contain the ♀ mutation under the cover of another normal allele, which will be disclosed in the progeny of this

TABLE 2

Methods used to distinguish between the mutations RT. HM. and ♀ HM., and ♂ HM. and ♀♂ HM. and HT. which give similar reactions in the ordinary tests



plant. These have been identified by testing the plants obtained by the X × P crosses. In RT. HM. mutation all the plants from this cross will be self-sterile, whereas in the case of ♀ HM., half the progeny will be self-fertile and the other half self-sterile.

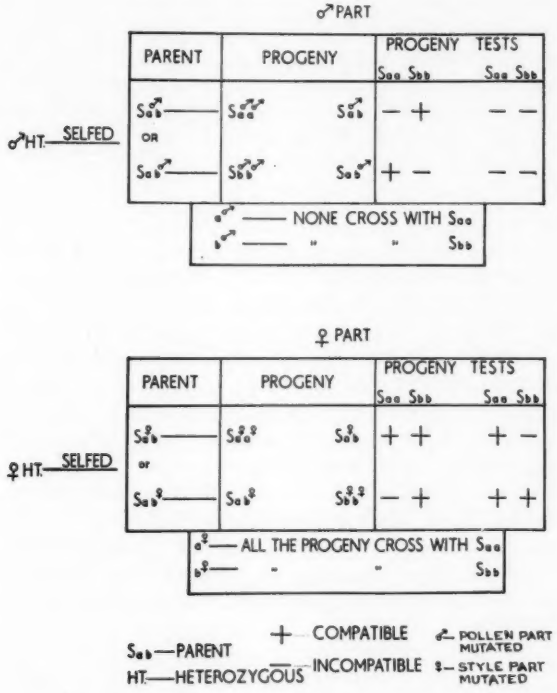
Identification of the mutated allele

Table 3 shows the technique used to determine which of the two parental *S* alleles has mutated. In ♀♂ mutations the whole gene (♂ as well as ♀ part) is changed and therefore these can not be homozygous or truly heterozygous (like the parent) for

TABLE 3

Identification of the mutated allele in plants containing ♂ HT. or ♀ HT. mutation

IDENTIFICATION OF THE MUTATED ALLELE



the parental *S* alleles. In these mutations it is also impossible to determine which of the *S* alleles has mutated. In other mutations, when only one of the two parts of the gene has mutated, the remaining unchanged part allows the identification of the affected allele. In the homozygous plants the mutated allele is directly known. In the heterozygous plants, however, it is identified by crossing the selfed progeny of these plants as female parent with the two test plants. In ♂HT. mutation half the progeny cross with neither of the test plants while the other half cross with one of them. The test plant which does not cross with any of the progeny identifies the mutated allele. In ♀ HT. mutation half the progeny crosses with both test plants whereas the other half crosses with only one of them. The test plant which crosses with all the progeny identifies the mutated allele. In RT. HT. mutation it is impossible to identify the mutated allele.

RATE OF MUTATION

Pollen estimation

Within species there is not much variation in the size of the stigma. Consequently there is no significant difference between various clones in the number of pollen grains sticking on the stigma after standard tripping. However, there is a significant difference between the two species *T. repens* and *T. pratense*, the former holding considerably more pollen on the stigma than the latter. This is due to the bigger size of pollen grains and not to the smaller size of stigma in *T. pratense*. The approximate diameters of pollen grains in *T. pratense* and *T. repens* are 0.049 ± 0.0014 mm and 0.028 ± 0.0022 mm, respectively. The number of pollen grains per stigma in *T. repens* and *T. pratense* is 356 ± 40 and 236 ± 52, respectively.

Dosage relationship

In order to select a particular dose of irradiation most suitable for this experiment, a test was carried out with one clone (2^T) of *T. repens* and four doses of irradiation, 750r, 1000r, 1500r, and 3000r. The effects of increasing dose on seed set, number of fully developed flowers per inflorescence and pollen sterility have been compared in table 4. Seed set is highest at 750r but decreases steadily with the rise in dosage. At 3000r no seed is recovered. The number of well-developed flowers per inflorescence is not seriously impaired until 1000r, after which it shows a sudden decline; at 3000r most of the inflorescences are either totally killed or develop only a few flowers. Pollen sterility steadily rises with the dose and reaches to about 50 percent at 1500r. As the increase in dose has a damaging effect on all the three factors related to the recovery of seed, the lowest dose of 750r was thought to be the best for this purpose. At this dose the mutation rate is higher than the control and damage to flowers and pollen fertility is the least. A higher dose would also adversely affect the germination of the recovered seed and the survival of the seedlings. Probably a slightly lower dose might have been still better.

Spontaneous mutation

T. repens produces on average 3.5 seeds per pod. In this species selfing, with or without irradiation, never produced more than 2 seeds per pod. Thus, it seems, most

TABLE 4

Effect of dosage on seed set, number of flowers per inflorescence and pollen sterility in the clone 2^T ($S_{6.7}$) *of* Trifolium repens

Character	Control	750r	1000r	1500r	3000r
Seed set per inflorescence	5.34 ± 3.14	7.25 ± 3.41	5.34 ± 2.68	3.79 ± 1.71	0.00
Number of flowers per inflorescence	70.25 ± 9.64	65.25 ± 12.52	67.00 ± 3.61	48.78 ± 10.72	Very few
Pollen sterility percentage	0.56 ± 0.10	18.32 ± 1.92	22.37 ± 2.90	48.20 ± 3.24	—

TABLE 5

Number of seeds set per 100 flowers selfed in various S *genotypes in* T. pratense *and* T. repens. *Control*

Genotype	Pl. no.	No. of flowers pollinated	No. of pollen grains tested $\times 10^6$	No. of seeds obtained	Seeds per 100 flowers	Seeds per million pollen grains approx.
			T. pratense			
$S_{2.4}$	32/1	1273	0.31 ± 0.07	5	0.39	16.1
$S_{1.4}$	32/9	545	0.12 ± 0.03	1	0.18	8.3
$S_{1.3}$	32/4	861		1	0.12	
	32/7	276 } 1830	0.46 ± 0.09	0 } 2	0.00 } 0.11	4.3
	32/13	693		1	0.14	
$S_{2.3}$	32/23	704	0.17 ± 0.04	14	1.99	82.3
$S_{5.7}$	33/3	272		0	0.00	
	33/4	604 } 876	0.21 ± 0.05	4 } 4	0.66 } 0.46	19.0
$S_{5.8}$	33/1	515		0		
	33/10	574 } 1089	0.24 ± 0.05	0 } 0	0.00	0.0
$S_{6.7}$	33/2	305		1	0.33	
	33/9	320 } 625	0.14 ± 0.03	0 } 1	0.00 } 0.16	7.1
$S_{6.8}$	33/7	872	0.19 ± 0.04	2	0.23	10.5
Swedish	250/4	241	0.05 ± 0.01	0	0.00	0.0
clovers	250/5	151	0.04 ± 0.01	0	0.00	0.0
	251/3	87	0.02 ± 0.005	0	0.00	0.0
	251/4	124	0.03 ± 0.006	19	15.32	633.3
	252/3	133	0.03 ± 0.006	0	0.00	0.0
	252/4	156	0.04 ± 0.008	6	3.85	150.0
			T. repens			
$S_{5.7}$	2^1	447	0.14 ± 0.02	5	1.12	35.7
$S_{6.7}$	2^7	1177	0.39 ± 0.04	72	6.12	184.6
$S_{5.6}$	2^7/10 (3)	433		4	0.92	
	(4)	197 } 630	0.21 ± 0.02	1 } 5	0.51 } 0.79	23.8
$S_{1.2}$	3^{20}	376	0.14 ± 0.02	0	0.00	0.0
$S_{2.3}$	3^{14}	514	0.18 ± 0.02	0	0.00	0.0
$S_{1.3}$	3^{20}/14 (10)	211	0.07 ± 0.008	1	0.47	14.3

of the pollen grains on the stigma containing a pollen reaction mutation have a chance to produce a seed in this species.

In *T. pratense*, however, the average seed set is only 1.1. Moreover, in this species, due to mechanical restriction in the style, normally only a few pollen tubes reach the bottom of the style. The chances of the recovery of pollen mutations are, therefore, very low in *T. pratense*. Under these conditions, mutation rates in the two species observed on the basis of the recovery of seed cannot be compared.

Table 5 gives the number of seeds obtained per 100 flowers selfed in various *S* genotypes in *T. pratense* and *T. repens*. In some genotypes two or more plants have been used for this experiment and their data have been given separately.

The mutation rate varies greatly among different *S* genotypes. Two ($S_{1.2}$ and $S_{2.3}$)

of the six genotypes in *T. repens* give no seed whereas one genotype ($S_{6.7}$) gives 6.12 seeds per 100 flowers selfed. Three others, $S_{1.3}$, $S_{5.6}$ and $S_{5.7}$, set 0.47, 0.79 and 1.12 seeds, respectively. In *T. pratense* those of the British origin mostly give a low seed set ranging from 0.11 to 1.99 seeds per 100 flowers. Only one genotype set no seed at all. Clovers of the Swedish origin, however, behave differently. Four out of the six selfed plants give no seed but the remaining two plants set considerably higher than the British clovers, i.e. 3.85 and 15.32 seeds per 100 flowers.

X-ray induced mutations

Table 6 gives the mutation rate in *T. pratense* and *T. repens* after X-ray treatment (750r). The effect of irradiation has been shown in table 7 by deducting the seed set in control from the seed set after irradiation.

TABLE 6

Number of seeds set per 100 flowers selfed after irradiation in T. pratense *and* T. repens

Genotype	Pl. no.	No. of flowers pollinated	No. of pollen grains tested × 10^8	No. of seeds obtained	Seeds per 100 flowers	Seeds per million pollen grains approx.
			T. pratense			
$S_{2.4}$	32/1	1555	0.38 ± 0.08	24	1.54	63.1
$S_{1.4}$	32/9	1439	0.33 ± 0.07	4	0.28	12.1
$S_{1.3}$	32/4	951		2	0.21	
	32/6	1453 } 2706	0.64 ± 0.14	3 } 5	0.21 } 0.18	7.8
	32/13	302		0	0.00	
$S_{2.3}$	32/23	722	0.16 ± .0.04	18	2.49	112.5
$S_{5.7}$	33/3	953 } 1635	0.38 ± 0.08	0 } 3	0.00 } 0.18	7.9
	33/5	682		3	0.44	
$S_{5.8}$	33/6	788 } 966	0.23 ± 0.05	1 } 1	0.13 } 0.10	4.3
	33/8	178		0	0.00	
$S_{6.7}$	33/9	1008	0.24 ± 0.05	6	0.60	25.0
$S_{6.8}$	33/7	587	0.14 ± 0.03	0	0.00	0.0
Swedish clovers	250/4	389	0.09 ± 0.02	1	0.25	11.1
	250/5	163	0.04 ± 0.008	0	0.00	0.0
	251/3	145	0.03 ± 0.007	0	0.00	0.0
	251/4	159	0.04 ± 0.008	14	8.81	350.0
	252/3	292	0.07 ± 0.01	2	0.68	28.6
	252/4	745	0.17 ± 0.04	24	3.22	141.1
			T. repens			
$S_{5.7}$	2^1	2337	0.82 ± 0.09	153	6.54	186.6
$S_{6.7}$	2^7	2736	0.96 ± 0.11	150	5.53	156.2
$S_{5.6}$	2^7/10 (1)	879		19	2.16	
	(3)	209		15	7.18	
	(4)	643 } 2171	0.78 ± 0.09	14 } 83	2.18 } 3.82	106.4
	(5)	440		35	7.95	
$S_{1.2}$	3^{30}	493	0.17 ± 0.02	2	0.41	11.8
$S_{2.3}$	3^{30}/14 (8)	170	0.06 ± 0.007	4	2.35	66.7
$S_{1.3}$	3^{30}/14 (10)	75	0.03 ± 0.003	1	1.33	33.3

TABLE 7

Number of seeds set per 100 flowers due to irradiation. This is determined by deducting the seed set in control from the seed set after irradiation

Genotype	Control seed set per 100 flowers	After irradiation seed set per 100 flowers	Seed set due to irradiation
	T. pratense		
$S_{2.4}$	0.39	1.54	+1.15
$S_{1.4}$	0.18	0.28	+0.10
$S_{1.3}$	0.11	0.18	+0.07
$S_{2.3}$	1.99	2.49	+0.50
$S_{5.7}$	0.46	0.18	−0.28
$S_{5.8}$	0.00	0.10	+0.10
$S_{6.7}$	0.16	0.60	+0.44
$S_{6.8}$	0.23	0.00	−0.23
Swedish clovers			
250/4	0.00	0.25	+0.25
250/5	0.00	0.00	0.00
251/3	0.00	0.00	0.00
251/4	15.32	8.81	−6.51
252/3	0.00	0.68	+0.68
252/4	3.85	3.22	−0.63
	T. repens		
$S_{5.7}$	1.12	6.54	+5.42
$S_{6.7}$	6.12	5.53	−0.59
$S_{5.6}$	0.79	3.82	+3.03
$S_{1.2}$	0.00	0.41	+0.41
$S_{2.3}$	0.00	2.35	+2.35
$S_{1.3}$	0.47	1.33	+0.86

In *T. repens* the response to irradiation is very conspicuous. Except in the case of $S_{6.7}$ where there is reduction in seed setting, all the five other genotypes show increased seed setting after irradiation. This increase is very marked in $S_{5.7}$, $S_{5.6}$ and $S_{2.3}$, i.e. 5.42, 3.03 and 2.35 seeds per 100 flowers, respectively. In $S_{1.2}$ and $S_{2.3}$ seed setting seems to be entirely due to irradiation as no seeds are produced in the control. In *T. pratense*, however, the response is of a different type. Although most of the genotypes —10 out of 14—show increased seed setting, the increase is generally very slight, ranging from 0.07 to 1.15 seeds per 100 flowers. Besides there are four cases of reduction in seed setting. This reduction is considerable (−6.51/100 fls.) in 251/4 (a plant of Swedish origin) where the seed set in control is very high (i.e. 15.32/100 fls.). Because the embryo sacs as well as the pollen mother cells are irradiated the increase in selfed seed set due to mutation of the *S* gene will be offset to some extent by the irradiation damage to the embryo sacs.

KINDS OF MUTATION

The reciprocal crosses in *T. repens* with ♂ mutations often showed a great difference in seed setting. This suggests that the pollen and style mutations may not be

TABLE 8

Elaboration of table 1, giving the reactions of complete or partial mutations in the two—"pollen" and "style"—parts of the S *gene. The number of plants obtained in different mutation groups have also been given. In* T. repens, *in 7 plants it was not determined whether they contained* ♂ *Hm. or* ♀ *mutation. These plants have been provisionally put in* ♀ *mutation group*

TESTING THE MUTATIONS

1	2						3						4						5		6		
TESTS	RT.																						
	HM.			HT.			HM.		HT.				HM.		HT.				HM.	HT.	HM.	HT.	
	N.	♂	♀	N.	♂	♀	N.		N.		♀		N.	♂	N.	♂							
X. SELFED	—	+	—	—	+	+	+		+		+		—	+	+	+			+	+	+	+	
P. × X.	—	+	—	—	+	—	+		+		+		—	+	—	+			+	+	+	+	
X. × P.	+	+	+	—	—	+	+		—		+		+	+	+	+			+	+	+	+	
NO OF PLANTS	1		2	3	1		9		1										1	3	24+7	1	PLANTS TESTED
T. REPENS TOTAL						7					10							0				31	52
NO OF PLANTS				10	1				2				7		1								
T. PRATENSE TOTAL			0			11					2							8		0		0	21

X. PLANT TESTED
P. PARENT
RT. REVERTIBLE
HM. HOMOZYGOUS
HT. HETEROZYGOUS
N. NORMAL

\+ COMPATIBLE
+ PARTLY COMPATIBLE
— INCOMPATIBLE

♂ COMPLETE POLLEN PART MUTATED
♂ PARTLY " " "
♀ COMPLETE STYLAR " "
♀ PARTLY " " "
♂♀ COMPLETE POLLEN AND STYLAR PARTS MUTATED
♂♀ PARTLY " " " " "

of the same degree; and the reciprocal crosses with the parent reveal the different degree to which the specificity has been lost. A number of plants were found where partial or full mutation of one part of the gene was accompanied by a slight change in the other part also.

Upon the above mentioned assumption, the reactions of table 1 have been further elaborated in table 8. All possible combinations of partial and complete mutations of pollen and style reactions have been given. Plants obtained due to mutation have been classified into various columns of this table.

Revertible mutations

In *T. pratense*, among the 24 plants (15 with irradiation, 9 without irradiation; representing the genotypes $S_{1.3}$, $S_{1.4}$, $S_{2.3}$ and $S_{2.4}$) grown from some of the selfed seeds obtained with or without the help of irradiation, 21 were self-sterile. As seeds could not have been produced without ♂ part mutation in the particular pollen affecting fertilization, it seems that the ♂ mutation was unstable and later on reverted to its normal state. One of these plants set a few seeds after selfing (14.5%). This indicates that either 'reversion' was incomplete or the original ♂ mutation was partial.

There were three plants, two from the selfed seeds (without irradiation) of the genotype $S_{2.3}$ and one from the selfed seeds (with irradiation) of the genotype $S_{2.4}$, (from 1.15 million pollen grains tested) which were completely self-sterile but crossed

reciprocally with their parents. As the explanation of these reactions will involve the assumption of the creation of an altogether new *S* allele—an assumption not supported by other results—it is thought that these plants may have arisen as a result of contamination. In *T. repens* no such plant was obtained among 52 plants tested (from 0.43 million pollen grains tested).

In *T. repens*, out of 52 plants tested, 7 were due to 'revertible' mutations; one RT. HT. mutation gave a few seeds after selfing showing that a partial pollen mutation was still present. The progeny test of 2 RT. HM. mutations showed that they contained a slight ♀ mutation, which could not be expressed in the X plants due to the covering effect of the normal allele.

♂ (*pollen reaction*) *mutation*

Three partly or fully self-fertile plants were obtained through irradiation in *T. pratense*. The self-fertility was due to ♂ mutations. These plants were heterozygous for *S* allele. In *T. repens* only one plant with ♂ mutation and heterozygous for *S* allele (♂HT.) was obtained. Nine more ♂ mutations with homozygous *S* alleles (♂HM.) were identified from the 39 families tested to distinguish them from ♀♂ mutations.

♀ (*style reaction*) *mutation*

If the ♀ mutation in the pollen is to be recovered it must be accompanied by the ♂ mutation. However, subsequent reversion of the ♂ mutation to the normal condition will leave only the ♀ mutation in the X plant. In *T. pratense*, in addition to one self-fertile ♀ HT. mutation, 7 other families, tested to distinguish between RT. HM. and ♀ HM. mutations, showed that all the 7 X plants had ♀ HM. mutations.

♀♂ (*both pollen and style reaction*) *mutation*

No such mutation was found in *T. pratense*, but in *T. repens* the majority of the self-fertile plants belonged to this (♀♂) reaction group. Out of 52 X plants tested, 31 (59.6%) contained both pollen and style mutations. These plants were self-fertile and crossed reciprocally with their parents. In *T. pratense*, which is a diploid, ♀♂ mutation may be lethal.

DEGREE OF SELF-FERTILITY

In the X plants the amount of seed set after selfing varies from a few seeds per 100 flowers to full compatibility. It is also seen that selfed or crossed progeny of X plants show a considerable variation with regard to self-compatibility and that their levels of self-compatibility are usually lower than those of their parents (table 9).

This apparent lower fertility on selfing in the 2nd generation may be due to the effects of inbreeding depression caused by the further generation of selfing. But in *Trifolium repens* three completely self-sterile plants were found in the second generation of selfing. The pollen fertility in these plants is high and they produce normal seed set on compatible crossing.

TABLE 9

Selfed-seed setting in X plants and their selfed progeny in T. pratense. *Average compatible seed set—110 seeds per 100 flowers*

Plant no.		No. of flowers	No. of seeds	Seeds per 100 flowers
X plants	Progeny			
X1/32 (3)		108	14	12.9
X4/32 (3)		25	28	112.0
	(1)	20	20	100.0
	(2)	41	39	95.1
	(3)	38	34	89.4
	(4)	23	17	73.9
	(5)	40	15	37.5
X1/32 (7)		142	110	77.4
	(1)	20	15	75.0
	(2)	31	22	70.9
	(3)	17	12	70.5
	(4)	28	17	60.7
	(5)	23	13	56.5
	(6)	29	14	48.2
	(7)	23	7	30.4
	(8)	20	4	20.0

TABLE 10

Rate of 'revertible' and permanent mutations in T. pratense *and* T. repens

Species	Total no. of plants tested	Total no. of pollen grains tested $\times 10^8$	'Revertible' mutation		Permanent mutation	
			No. of plants	Mutation rate per million pollen grains approx.	No. of plants	Mutation rate per million pollen grains approx.
T. pratense (with irradiation 750r)	15	0.61 ± 0.155	7	11.5	8	13.1
T. repens (spontaneous)	18	0.26 ± 0.029	5	19.2	13	50.0
T. repens (with irradiation 750r)	32	0.23 ± 0.26	2	8.7	30	130.4

NATURE OF MUTATIONS

Table 10 gives the rates of 'revertible' and permanent mutations in the two species. There is considerable difference between the two species in the proportion of 'revertible' and permanent mutations. In *T. repens*, with irradiation, most of the mutations (94.25%) are permanent whereas in *T. pratense* the rates of the two kinds of mutations are approximately equal. In *T. repens*, in the control, the rate of permanent mutation is more than double the rate of 'revertible' mutation. With irradiation the rate of 'revertible' mutation decreases to a negligible proportion, whereas the rate of permanent mutation is very high. This shows that irradiation not only increases the general mutation rate but that it also helps to convert most of the spontaneous 'revertible' mutations into permanent ones.

TABLE 11

A summary of the different kinds of mutation in the various genotypes of T. pratense *and* T. repens. *Mutability of the different* S *alleles is also given*

Species and genotype	Total no. of plants tested	'Revertible' mutations	Permanent mutations		Mutated allele			
			Complete (♂♀) mutations	Part (♂ or ♀) mutations	No. of plants with known mutated allele	Allele	No. of plants	Part mutated
T. pratense								
$S_{2.4}$	10	6	—	4	4	S_2	3	♀
						S_4	1	♂
$S_{1.2}$	5	2	—	3	3	S_1	2	♀
						S_2	1	♀
$S_{1.4}$	4	2	—	2	2	S_1	1	♀
						S_4	1	♂
$S_{2.3}$	1	—	—	1	1	S_2	1	♀
T. repens								
$S_{6.7}$ (spontaneous)	18	5	7	6	8	S_6	7	♂
						S_7	1	♂
$S_{6.7}$ (with irradiation)	22	—	17	5	5	S_6	2	♂
						S_7	1	♂

The difference between the two species regarding the rates of 'revertible' and permanent mutations may be due to the one, *T. pratense*, being a diploid and the other, *T. repens*, a tetraploid. Permanent loss of the incompatibility reaction, particularly when it affects both ♂ and ♀ parts of the *S* gene, is probably lethal. In the tetraploid, lethal effects of the deleterious mutations are more likely to be covered by other chromosomes whereas a diploid is exposed to such harmful effects.

Table 11 gives a summary of the different kinds of mutation in various genotypes of *T. repens* and *T. pratense*. The mutability of the different *S* alleles has also been given.

The kind of mutations in different alleles differs in the two species. In *T. repens* all the permanent mutations are pollen mutations whereas in *T. pratense* most of them are stylar mutations. Only the allele S_4 gives pollen mutations; the other three alleles, S_1, S_2 and S_3, give only stylar mutations.

In *T. repens* the permanent mutation in S_6 occurs spontaneously only in the pollen reaction, but with irradiation generally the whole gene (pollen and stylar parts) is permanently changed. Thus the recovery of the pollen mutations is lower with irradiation than that produced spontaneously (table 11).

It seems that generally both parts of the *S* gene (pollen and stylar) are simultaneously affected but the sensitivity of the two parts is different. This leads to a permament mutation of one part only, both of them, or none.

In *T. repens* it is the ♀ part which is less affected and thus reverts to its original state, but in *T. pratense* it is generally the ♂ part which reverts. In both species the two parts of the same allele behave consistently with regard to the response to mutagenic agencies. In *T. pratense* the alleles S_1 and S_2 always show ♀ mutation and the

allele S_4 ♂ mutations. Similarly, in *T. repens* the two alleles, S_6 and S_7, always show ♂ mutations only.

AUERBACH (1950) suggests an explanation for 'revertible' mutations. On the evidence of mosaics produced by chemical mutagens she assumes that sometimes these agencies may produce labile premutation which subsequently, after one or more cell divisions, may give rise to the actual mutation or may revert to the old allelomorph. Cytological basis for such assumption is suggested by DARLINGTON (1950), who thinks that the 'revertible' mutation is due to the sorting out of mutated from unmutated polypeptide chains composing the chromosomes.

Recently LA COUR and RUTISHAUSER (1953) reported sub-chromatid breakage by irradiation during prophase stage in *Scilla sibirica* endosperm but not during resting stage and metaphase. These authors point out the probability of a causal relationship between sub-chromatid breakage and delayed mutations. This is further supported by LEWIS and CROWE (1953) who obtained a 'revertible' mutation in Prunus with irradiation of prophase; only stable 'complete' mutations were discovered with treatment of the resting stage.

INHERITANCE OF SELF-FERTILITY

Self-fertile plants have been obtained from selfing self-compatible plants containing self-fertility (S_f) allele. Plants with S_f alleles have been discovered in *Nicotiana langsdorffii* (EAST 1929), *Trifolium pratense* (WILLIAMS and SILOW 1933; WILLIAMS and WILLIAMS 1947) and *Trifolium repens* (ATWOOD 1945). These plants bred true for self-fertility.

However, a number of cases have been reported where self-fertile plants were produced as selfed progeny of self-incompatible plants. Such self-fertile plants were obtained in *Cardamine pratensis* (CORRENS 1913), *Secale cereale* (HERIBERT-NILSSON 1916), several species of Lilium and Linaria and also in *Cichorium intybus*, *Brassica pekinensis*, *B. chinensis* and *B. oleracea*, var. *capitata* (STOUT 1917, 1918, 1927; KAKIZAKI 1930). Selfed progeny of these self-fertile plants gave both self-fertile as well as self-sterile plants. STOUT (1927), after several years of continued selection for self-fertility, was neither able to secure any appreciable increase in the relative number of self-fertile plants nor to isolate any strain that continued to be self-fertile.

Data on self-fertile plants obtained as selfed progeny of self-incompatible plants suggest that there may be two kinds of self-fertility involved: (1) self-fertility due to mutation and (2) self-fertility, in plants with sporophytic control, due to dominance relationship between the *S* alleles.

Self-fertility due to mutation

In the present experiments with Trifolium spp., self-fertile as well as self-sterile plants were obtained as selfed progeny of self-incompatible plants. Self-sterile progeny are produced as a result of 'revertible' mutations (LEWIS and CROWE 1953). Self-fertility is due to partial (♂ or ♀) or complete (⚥) mutation in the *S* allele. These self-fertile plants, like self-fertile plants with S_f allele, generally breed true for self-fertility. However, many of the self-fertile plants obtained from selfed self-incompatibles were only partially self-fertile. These, and also some of the nearly fully self-

fertile plants, gave selfed progeny which showed various grades of self-fertility. All the progeny, however, showed lower self-fertility than the parent. In a few selfed families, where the mother plants were only partially self-fertile, a few completely self-sterile plants were also obtained along with other partially self-fertile plants. These self-sterile plants did not show any kind of inbreeding depression and were perfectly normal.

Various grades of self-fertility encountered in these experiments may be due to (1) the different degrees of mutation (partial mutation) and (2) the segregation of compatibility promoting modifiers. It seems, when partial mutation greatly weakens the incompatibility but does not completely remove it, the minor modifying genes further weaken or strengthen the reaction according to the number of such genes present and their chance recombinations in pollen grains. But when mutation is complete the small effect of the incompatibility promoting modifiers have no consequence. The effect of modifiers has also been observed in tetraploid red clover when the incompatibility reaction is greatly reduced but not entirely eliminated (PANDEY 1956). EAST and YARNELL (1929) believed that subsidiary factors at another locus than the *S* in Nicotiana spp. had effects upon the rapidity of pollen tube growth.

It is, therefore, possible to recover a few completely self-sterile plants among the selfed progeny of self-fertile plants. Thus, some of the cases where self-fertile as well as a few self-sterile plants are obtained in the selfed progeny of self-fertile plants, and where the incompatibility system is definitely established to be typically gametophytic, Nicotiana type, can be satisfactorily explained on the above assumptions. However, it is possible that all the progeny of a cross between two self-fertile (S.F.) plants may be fully self-sterile (S.S.):

$$\underset{S\,a\,b}{\underset{(S.F.)}{\overset{\varphi}{}}} \times \underset{S\,a\,b}{\underset{(S.F.)}{\overset{\varphi}{}}} \rightarrow \underset{S\,a\,a}{\underset{(S.S.)}{\overset{\varphi}{}}} + \underset{S\,a\,b}{\underset{(S.S.)}{}}$$

Self-fertility due to dominance relationship between the S alleles

Recently a system of incompatibility has been described in two homomorphic species of Compositae which is based on sporophytic determination of both pollen and style reactions (HUGHES and BABCOCK 1950; GERSTEL 1950). In these species *S* alleles show dominance or individual action according to the combination in which they are found. CROWE (1954) reported the same system of incompatibility in *Cosmos bipinnatus*. In this species she found two alleles which acted individually in the style but showed dominance in the pollen. This indicates that in certain combinations of *S* alleles dominance relationship may be different in the style and the pollen. If it is supposed that in certain combinations one allele is dominant in the style and the other in the pollen, the plant will become self-fertile. Half the progeny of such self-fertile plants will be self-fertile and half self-sterile.

Assuming there were other minor genes which modified the expression of dominance, their segregation will bring about differences in self-fertility in the selfed progeny. However, no amount of selection for self-fertility is likely to yield a purely self-fertile strain in these plants.

STOUT's (1927) inability to fix a pure self-fertile strain after several generations of selection in *Cichorium intybus*—which also belongs to Compositae—may be due to such a combination of *S* alleles. In Cruciferae (CORRENS 1913; STOUT 1927; KAKIZAKI 1930) also, where such type of self-fertility has been reported, it seems very likely that self-fertility may be due to a similar situation.

SUMMARY

The incompatibility genes in the two species, *Trifolium repens* (2n = 32) and *T. pratense* (2n = 14) have been found to mutate both spontaneously and by X-irradiation.

Four kinds of mutations have been identified: (1) revertible mutation, (2) pollen reaction mutation, (3) style reaction mutation, and (4) both pollen and style reaction mutation.

The two species differ in their rates of 'revertible' and permanent mutations. With irradiation the two types of mutations occur in approximately equal proportions in *T. pratense*, but in *T. repens* most of the mutations (94.25%) are of a permanent nature.

No mutations in which both the stylar and pollen reactions had been changed were found in *T. pratense*, but about 60% of the mutations in *T. repens* were of this double type.

Different alleles have different rates of mutation in their "pollen" and "style" parts.

With the exception of three cases in *T. pratense* which were probably due to contamination all the mutation changes were to self-fertility.

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STUDIES OF THE GENETIC VARIABILITY IN WILD POPULATIONS OF HOUSE MICE. I. ANALYSIS OF SEVEN ALLELES AT LOCUS T[1]

L. C. DUNN AND JOAN SUCKLING

Columbia University, New York City

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GENETICAL polymorphism, the occurrence of several genotypes in the same population, is now recognized as the normal state of the population structure of many crossbreeding species in nature. The maintenance of the genetic variance in a state of equilibrium, as in balanced polymorphism, has been ascribed in a number of cases to the superior fitness of heterozygotes. DOBZHANSKY, (1949) for example, has shown that most of the chromosomes of wild populations of several Drosophila species contain genes which are lethal or otherwise deleterious when homozygous.

Little has been known about such genetical conditions within wild populations of mammals, largely due to the difficulties of analyzing the genotypes of wild mammals in the laboratory. These difficulties have now been overcome with one species, the house mouse (*Mus musculus*), to a sufficient extent to permit rapid diagnosis of the conditions at one locus in animals taken from wild populations. The methods of doing this have been described (DUNN and MORGAN 1953), and the results of a preliminary survey (DUNN 1955) indicate that the majority of wild populations from several parts of the U.S. contain heterozygotes for different genetic variants at this locus.

In addition, two possible mechanisms contributing to the maintenance of heterozygosity in these populations have been suggested by the evidence. One is superior fertility of heterozygotes (DUNN and SUCKLING 1955); the other is a novel means, discovered in this material, by which gametes carrying the variant allele, are transmitted in higher than normal frequency by male heterozygotes. The latter, which we have called segregation ratio advantage (DUNN 1953) appears to be a component additional to those usually considered in explaining the equilibria characteristic of wild populations; namely, mutation rate, selection or fitness, migration and random drift. The genetical state of a wild population may thus be conceived as the balance reached through the interactions of these five elementary agencies with a particular environment. The wild mouse populations provide an opportunity of evaluating certain of these, particularly the segregation ratio effect at the one locus at which it has been identified. Estimation of the other parameters will be more difficult in mammals, because of the long generation interval and the slow development, but the latter, when aided by embryological methods, may turn out to be an advantage, since the components of fitness—such as fecundity, fertility, viability of embryos and of suckling young, growth, life-length and the like—may be studied separately, and the effects of individual genes upon them elucidated. For the mouse there is already much information of this sort (cf. GRÜNEBERG 1951).

[1] Based on breeding experiments carried out at Nevis Biological Station, Irvington-on-Hudson, N. Y., with support from the Higgins Trust, Columbia University and the Rockefeller Foundation. The assistance of DR. W. C. MORGAN, JR., in the early part of this work is gratefully acknowledged.

WILD MOUSE POPULATIONS

The house mouse is a commensal, its geographical distribution corresponding closely with human habitation. While its spread over both hemispheres has probably been due in the main to human agencies—shipping and the like—and while most populations are found in dwellings and places of food storage, there are nevertheless many free-living populations remote from habitations. These are presumably feral, originating from commensal populations and possibly in reproductive communication with them. Most of the populations studied up until now appear to be small—from a few individuals to a few hundred—and the small amount of data available indicates that each population is limited to a rather restricted home range (cf. BLAIR 1953). It is probable that the species now consists of a large number of small populations, each occupying its own ecological niche. The factors influencing reproductive isolation of separate populations are probably those which operate on mammalian populations generally, but in the case of the mouse, some are probably connected with the human occupation pattern. The populations can thus be conceived as "natural" ones only in a limited sense. The species can be treated as intermediate between such domesticated populations as have no known ancestral population in its natural state (e.g., chickens, dogs) and those living in independence of man. We shall refer to "wild" mouse populations to distinguish them from those bred in the laboratory or by fanciers, without implication as to their natural state. In collecting from wild populations we, of course, avoid the immediate neighborhood of populations of laboratory or domesticated mice, but there is no way of knowing, except from the study of the wild mice themselves, what their connections with domestic populations may have been.

METHODS OF STUDY

Mice are caught individually in traps (we have used the "Hav-a-Hart" from Albia Products Co., New York) baited with peanut butter. The point at which each mouse is caught is noted on the trap when collected, if out-of-doors by landmarks, if indoors by identification of building and room. In the laboratory matings are arranged (by inbreeding or testcrossing) in such a way that the "wild" genes of any descendant trace to a particular wild individual. Wild females are mated in pens with exercise wheels which tend to induce and maintain oestrus, although even with this treatment some wild females have remained in anoestrus. Wild males do not require this and breed well with domestic females in ordinary steel mouse boxes ($6'' \times 6'' \times 12''$) with wire mesh tops from which feed wells depend into the pens. Wild mice, like the laboratory stocks, are fed the usual ration of pellets (Rockland Farms) supplemented by green feed pellets (Full O' Pep), rolled oats and ground wheat with water from drip bottles. Litters from wild females are usually given to domestic females for fostering.

BREEDING ANALYSIS

Although the samples from most wild populations show evidence of being polymorphic in several noticeable characters (white belly-yellow belly-gray belly; white spotting on forehead, belly or tail; different densities of black pigment in the agouti

pattern) we have as yet studied only the variants at locus *T*. Each wild mouse is first testcrossed with mice of genotype $T/+$ from a standard laboratory stock of this mutant (Brachy = short tail). If tailless mice are born from this mating, these are then tested to determine whether they contain an allele of the series collectively known as t^w, which in compound with $T(T/t^w)$ produced taillessness, or whether they are $T/+$ with minus modifiers of tail-length. The latter have been found in several wild populations. Such tests are carried out by mating F_1 tailless from a wild parent (1) with each other (2) with tailless laboratory stocks known to be T/t. If the test crosses yield Brachy ($T/+$) offspring, the F_1 tailless is classified at $T/+$ and discarded. If only tailless or tailless and normal-tailed offspring are produced, the F_1 tailless is tentatively diagnosed as T/t^w. If the testcross by a domestic stock such as T/t^0 yields only tailless, then the t^w allele from the wild is diagnosed as either the same as the domestic test allele or lethal in compound with it (e.g. t^w/t^0). Only one such case has occurred in our tests. If inter-se matings of F_1 tailless yield only tailless offspring, while test matings with a domestic allele yield both tailless and normal, the allele from the wild is diagnosed as a lethal which forms a balanced lethal stock in compound with *T*; that is, $T/t^w \times T/t^w$ give as viable zygotes only T/t^w. If inter-se matings of F_1 tailless yield tailless *and* normal offspring the allele from the wild is diagnosed as viable, and the normals are then assumed to be t^w/t^w, an assumption which can be tested by mating them with $T/+$. If the assumption is correct this cross should yield only tailless (T/t^w) and normals ($+/t^w$) and never any Brachys ($T/+$). Inbred stocks, each containing a different wild allele $T/t^{w1\cdots n}$ are then set up and different balanced lethal lines are then cross-tested with each other. If the cross $T/t^{w1} \times T/t^{w5}$, for example, yields both tailless (T/t^{w1} and T/t^{w5}) and normal offspring the two t^w alleles are assumed to be different and the normals are assumed to be t^{w1}/t^{w5}, an assumption which can be tested by mating them with Brachy as outlined above. If the cross $T/t^{w5} \times T/t^{w6}$ (for example) yields only tailless, then the alleles are assumed to be either the same, or to be lethal in compound. The latter assumption can only be tested by identifying as dead zygotes during embryogeny those assumed to be t^{w5}/t^{w6}. Viable alleles cannot be distinguished directly as the same or different by the above methods. Here recourse must be had to another criterion. Male compounds containing a lethal and one viable allele, e.g. t^0/t^{w2} (t^0 lethal, t^{w2} viable) may be completely sterile or quasi-sterile, while with a different viable allele, t^7, the compound is fertile (DUNN 1952). This test is so laborious that it is resorted to only in cases of great importance. The result is that for practical purposes viable alleles from different populations cannot be differentiated, and the tests are routinely applied only to lethal alleles.

RESULTS

Preliminary results of the analysis of the first four alleles found in wild populations have been published (DUNN and MORGAN 1953). Of the four alleles identified three were lethal and one viable. One of the lethals could not be distinguished from the domestic allele t^0, and was not studied further. Additional data on the first three are reported here together with studies of three new alleles identified in three new and different wild populations. The basic data on the seven alleles are set out in table 1.

TABLE 1[1]

Results of breeding analysis of T*-alleles identified in wild populations*

Allele	Source	Testcross wild $+/t^w \times T/+$			Tailless × Tailless $T/t^w \times T/t^w$			Tailless ♂ × normal ($T/t^w \times +/+$)		Ratio t^w
		Normal	Brachy	Tailless	Tailless	Normal	Litter Size	Normal	Brachy	
t^{w1}	N.Y.1(1♂)	23	2	20	206	(10)*	2.90	262	49	.84
t^{w2}	N.Y.1(1♂)	80	3	54	355	235	5.67	319	15	.95
t^{w3}	Conn.1(1♀)	10	4	3	261	(3)†	3.77	379	4	.99
t^{w4}	Wisc.3(1♂)	16	1	10	19	—	4.75	187	5	.97
t^{w5}	N.Y.2(1♂)	5	1	2	368	1‡	3.77	446	30	.93
t^{w6}	Fla.2(1♂)	3	—	2	212	—	3.65	90	1	.99
t^{w7}	Tex.1(1♂)	24	5	17	75	71	4.86	212	15	.93

[1] We are indebted, for specimens collected and sent to us, to: DR. HOWARD SCHNEIDER (N.Y. 1); MR. W. C. MORGAN, SR. (Conn. 1); MR. CHARLES SOUTHWICK (Wisc. 3); MISS NAOMI FITCH (N.Y. 2); MR. SIDNEY HOLMAN (Fla. 2) and DR. W. FRANK BLAIR (Tex. 1).

* 10 dead at birth or stillborn, 9 with microcephaly.

† 3 stillborn, 1 with microcephaly.

‡ Viable exception, quasi-sterile male t^{w5}/t^z.

The results of testing the original wild heterozygote from which each allele was derived are shown in columns 3, 4 and 5. In all except one case this was a male, and the results show clearly the unequal ratio of offspring derived from the +gamete (15 Brachy) and from the t^w gamete (108 tailless). This is a constant feature of all male heterozygotes from wild populations and since it is usually apparent in the first litter, male heterozygotes will usually be diagnosed first and the new alleles extracted from them. The population samples tested were all small: 12 males in the case of N.Y. 1 of which seven were $+/t^w$ and five $+/+$; three females from Conn. 1 of which one was $+/t^w$; six males from Wisconsin (a composite sample from several sources near Madison) of which one male was from a population inbred in confinement from a wild source; this male was $+/t^w$; a single male from N.Y. 2 (W. 106th Street) which escaped after spending one night in the laboratory, during which time he mated with a Brachy female and proved to be $+/t^w$; 4 males from Florida 2 (Sarasota) of which one proved to be $+/t^w$; six males and four females from a Texas population (near Austin) of which three males and one female were $+/t^w$. In this case the alleles extracted from all four heterozygotes were tested and behaved alike, as viable when homozygous. We assumed all four to be the same allele and saved only that derived from the first male. One population (N.Y. 1) yielded two different alleles, one lethal and one viable; but this population was of composite origin, descended from a mixed sample from New York City and Philadelphia, Pa., which had been inbred for eight years in the laboratory of DR. HOWARD SCHNEIDER of the Rockefeller Institute for Medical Research before we tested it. Although this is the only case in which two different alleles were found in the same sample, the numbers of animals tested from other samples were too small to give any estimate of the likelihood that single populations contain more than a single allele. Larger samples from additional populations are now being tested with this important point in mind.

The data are also inadequate to give good estimates of the frequency of heterozygotes in wild populations. Combining the above data with those from five additional populations in which heterozygotes have recently been found leads to an estimate of about 50 percent as the average frequency of heterozygotes in such populations (DUNN 1955).

The results of inter-se matings of tailless offspring derived from single wild heterozygotes are shown in columns 6, 7 and 8. The alleles t^{w2} and t^{w7} are diagnosed as viable on the basis of the inter-se matings and on progeny tests of the normals both from such matings, which prove to be t^{w2}/t^{w2} and t^{w7}/t^{w7} respectively. Females of these genotypes produce only tailless (T/t^{w}) and normal t^{w}/t^{w} when tested by Brachy ($T/+$) and never give $T/+$ offspring. Males homozygous for either of these alleles are generally sterile although we have found one quasi-sterile male of genotype t^{w2}/t^{w2}.

Alleles t^{w1}, t^{w3}, t^{w4}, t^{w5} and t^{w6} are diagnosed as lethals on the behavior of the tailless lines T/t^{w} as balanced lethal systems. Matings of T/t^{w1} produced in the early generations 10 exceptional animals, all dead at birth or stillborn, nine of them abnormal with small heads and failure of development of the eyelids. This suggested that they might be t^{w1}/t^{w1} and that death of such homozygotes might occur near the time of parturition. No such exceptions have been noted in the last six generations and the point has not been investigated embryologically. Three similar exceptions occurred in the first two generations of T/t^{w3} matings but none since. Matings of T/t^{w5} produced a single exceptional normal male in the sixth generation. This male was quasi-sterile, probably of genotype t^{w5}/t^{wx}, although the new allele assumed to have arisen by mutation (cf. similar cases in DUNN and GLUECKSOHN-WAELSCH 1953) has not yet been isolated. Matings of T/t^{w6} have given only the results expected from a balanced lethal line.

Comparison of litter sizes confirms the above diagnosis. The average size of 301 litters from balanced lines containing lethal t^{w} alleles was 3.58. The average size of 134 litters from lines containing viable t^{w} alleles was 5.49. If we assume the former were reduced before birth by 50 percent (two lethals) and the latter by 25 percent (one lethal, T) then the unreduced litters in the first case would average 7.16, and in the second 7.32, i.e. comparable sizes.

Segregation ratio abnormality in wild heterozygotes

One of the most striking features of the results of testing wild populations is the very unusual outcome of all test crosses of $+/t^{w}$ males by $T/+$ females. The expected results are, of course, 50 percent normal ($+/+$ and $+/t^{w}$) 25 percent Brachy $T/+$, and 25 percent tailless T/t^{w}, and these results are obtained when $+/t^{w}$ *females* are tested by $T/+$. But when $+/t^{w}$ *males* are tested, the ratio of Brachy to tailless, which expresses the segregation ratio of $+$ to t^{w} gametes, is never 1 to 1. From tested male wild heterozygotes the ratio of Brachy to tailless was 16 to 108; that is, about 87 percent received the t^{w} allele.

That the ratio is a genotypic one, rather than due to some phenotypic disturbance, was shown in one case by testing all of the available normal offspring of one $+/t^{w2}$ male which had been mated to $T/+$ females. Out of 46 tested, 42 (91%) proved to

be $+/t^w$, showing that the abnormal ratio found between the Brachy and tailless classes also obtains within the phenotypically normal class.

The chief evidence on segregation ratios of alleles derived from the wild consists of the results of testcrosses of tailless males T/t^w by normal $+/+$ females from our standard inbred ($T/+$) stock (table 1, columns 9, 10 and 11). This cross produced two phenotypic classes, normal and Brachy. The former outnumber the latter by about 9 to 1. Large samples of the normal-tailed class have been progeny-tested and always prove to be $+/t^w$. We have, therefore, assumed that there is an abnormality either in the segregation mechanism by which T and t^w sperm are produced, or by which these two types of sperm function in fertilization to very different degrees. For the purposes of a first analysis we assume the former; namely, that the gametic ratio of t^w to T is abnormal, and that t^w sperm are produced in great excess. The ratio of t^w to T varies somewhat in different males (as deduced from progenies) but is always in excess of .8 and is generally over .9.

It is not clear from the present evidence whether variations in the ratios are determined by the properties of the individual alleles, or whether the ratios are affected by factors external to but acting on the t-alleles. These hypotheses can be tested first by comparing the ratio abnormalities of a series of different alleles when placed in the same isogenic background; second by comparing the ratios of the same allele in different genotypic backgrounds. These tests have not been made for the wild alleles. Partial studies of some domestic alleles give some indication that each allele has its characteristic ratio (DUNN and GLUECKSOHN-WAELSCH 1953) which, however, may be modified somewhat by selection. The fact that all alleles in wild populations give extremely aberrant ratios in favor of t^w suggests that other factors in the wild genotypes, which tend to maximize this ratio, have some selective advantage under wild conditions; and, thus, that the ratio abnormality itself or the great excess of heterozygotes to which it leads, is advantageous. It may be that effects of t-alleles on the meiotic mechanism itself, of which the ratio change is the expression, is the property responsive to natural selection.

In the wild populations, the ratio abnormalities actually found must confer an enormous advantage on the t^w alleles as compared to the normal alleles. This is sufficient to counterbalance the selection against the t^w lethals and the t^w viable alleles which are male sterile (PROUT 1953). The equilibrium values to be expected from different values of the ratio factor, when opposed by selection due to lethality, are being worked out.

Preliminary results (to be published elsewhere) indicate that for a segregation ratio advantage of about .8, the equilibrium value for t^w heterozygotes is near to 50 percent and this is about the average value found in samples from the first 12 wild populations tested (DUNN 1955). However, these computations have been based on random mating in large populations, whereas wild populations are probably small and hence inbred; and theory has still to be developed for such models.

The cause of this ratio abnormality is not known. One hypothesis being tested is that in spermatogonial stages of $+/t^w$ males, t^w induces a mutation or conversion of the $+$ allele to t^w, resulting in a mosaic gonad consisting of large sections t^w/t^w and smaller sections $+/t^w$. Other hypotheses are, of course, possible, but those upon which

some evidence has been obtained, such as post-reduction reduplication of t^w cells, have not seemed promising (DUNN and GLUECKSOHN-SCHOENHEIMER 1939). It must not be forgotten that some of the *t*-lethals may be connected with a chromosomal rearrangement and this may introduce irregularities into spermatogenesis. Too little is known of the cytogenetic situation in this chromosome to permit evaluation of this possibility.

Identity of lethals from different wild populations

A question of primary importance is whether different alleles are found in different wild populations and whether the wild alleles differ as a group or individually from any of the alleles which have arisen under observation in the domestic stocks of the laboratory which have been shown to be of mutative origin. Of the latter, eleven have been studied as follows: *T*, a lethal with dominant effect on the tail; t^0, t^1, t^4, t^9 and t^{12} are lethals shown to be different from each other, since they form viable compounds with each other; t^3, t^7, t^8 and t^{13} are viables of which t^3 is probably different from t^7 and t^8, the other relations being untested.

All seven of the wild alleles have been tested by t^0 and t^1. Of the lethals, only t^{w4} gives no evidence of being different from t^0 by the compound viability test. Fifty offspring from $T/t^0 \times T/t^{w4}$ were tailless; so the compound t^0/t^{w4} may be lethal. Of intercrosses among wild lethal alleles only t^{w5} and t^{w6} have failed to produce viable compounds at birth. Embryonic stages of intercross hybrids of $T/t^{w5} \times T/t^{w6}$ are now being examined. The cross $T/t^{w1} \times T/t^{w3}$ has not yielded sufficient offspring to permit a decision on these. At present it can be said that t^{w1} is different from t^{w2}, t^{w4}, t^{w5}, t^{w6} and t^{w7}; t^{w2} is different from all others except possibly t^{w7}, the other viable allele; t^{w3} is different from t^{w2}, t^{w4}, t^{w5} and t^{w7}; t^{w4} has been insufficiently tested and because of its similarity to t^0 has been discarded; t^{w5} is different from t^{w1}, t^{w2}, t^{w4} and t^{w7}; t^{w6} is different from t^{w1}, t^{w2} and t^{w7}.

It is clear that there is a variety of alleles in different wild populations and that most of these are different from at least two of the domestic alleles.

THE MANNER OF ORIGIN OF WILD t^w ALLELES

The seven alleles which were observed at origin in laboratory stocks all arose as exceptions from the balanced lethal line T/t^1; similar exceptions have been observed in the line T/t^0 but were either lost or were sterile (DUNN and GLUECKSOHN-WAELSCH 1953). A similar exception was found in line T/t^{w5}. This was a male, quasi-sterile as some other analysed exceptions have been and hence compatible with the hypothesis that it was t^{w5}/t^{wx}. Because of the sterility, no line has yet been derived from this exception so the hypothesis is untested. The fact that all new alleles observed at origin were from lines which already contained a *t*-allele, suggests that they arose by mutation either from a pre-existing *t*-allele, or were induced by the *t*-allele in the partner chromosome. The latter hypothesis is being tested in an experiment now in progress. No evidence of recombination in these balanced lethal lines has been found (DUNN and GLUECKSOHN-WAELSCH 1953).

If the origin of a new *t*-allele thus depends on the prior presence of a *t*-allele, how are we to explain the origin of the first one which induced the later ones, and how are

we to explain the multiplicity of different alleles in the wild populations? It is a noteworthy fact that no case of origin of a new *t*-allele has been observed in the Brachy ($T/+$) stock which has been inbred in the laboratory in large numbers since 1932. A few exceptional tailless animals have been found in this line but all of those tested have proved to be $T/+$, probably with modifiers at other loci. Mutation of either T or $+$ to t would be readily detected in this stock. If T and $+$ are relatively stable alleles, while the combination T/t is unstable, the cause of instability is probably connected with the nature and effects of *t*-alleles.

In the wild populations in which $+/t^w$ heterozygotes are found we may infer that t^w has arisen either by mutation of $+$, of which no instance has been found, or more probably that the t^w allele has arisen from an antecedent t^w in that population or in an ancestral one. Some chainwise connection between t^w alleles is thus suggested and with it a possible method for tracing relations between populations. This would only be valid if it could be shown that $+$ alleles from populations shown not to contain a t^w allele are in fact stable. This point is being tested by crossing such $+/+$ animals from wild populations by $T/+$, attempting to detect changes from $+$ to t^w. At the rate of mammalian reproduction, even in mice, this will require a long time.

SUMMARY

Evidence is presented on the breeding behavior of seven alleles t^{w1}–t^{w7} at locus T in the house mouse, each allele being derived from a single heterozygote caught in a wild population. Five of the alleles were lethal before birth, two formed viable homozygotes. All alleles produced a tailless phenotype when combined with the mutant T and none showed recombination with T.

Each allele tested showed normal segregation in T/t^w and $+/t^w$ females, but T/t^w and $+/t^w$ males always produced a great excess of offspring which inherited the t^w allele. This is attributed to an abnormality in the segregation mechanism leading to ratios of t^w sperm from .8 to .99, usually above .9. This gives the t^w alleles a great advantage and permits the lethals to be maintained in wild populations, in which up to 50 percent of individuals are heterozygotes. There may be in addition a selective advantage accompanying the heterozygote state.

Lethal alleles occurring in different wild populations were shown in several cases to be different alleles, compounds with different alleles such as t^{w3}/t^{w5} being viable. Several of them were also shown to be different from alleles previously found to have arisen by mutation in balanced lethal lines maintained in the laboratory (domestic alleles t^0–t^{13}). A considerable variety of allelic transformations at this locus is thus indicated.

The causes of the segregation ratio abnormality and the manner of mutation at this locus are unknown but it is suggested that they are connected, possibly through the induction of changes in the normal allele by an existing *t*-allele.

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INCOMPATIBILITY IN AUTOTETRAPLOID TRIFOLIUM PRATENSE

K. K. PANDEY

John Innes Horticultural Institution, Bayfordbury, Hertford, Herts.

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TRIFOLIUM pratense L. is a highly self-incompatible species. An oppositional system of incompatibility has been established in this species by WILLIAMS and SILOW (1933). Artificially induced autopolyploids have been produced by LEVAN (1948) and FRANDSEN (1948) and selections have been carried out for several generations to improve their fertility. But no work has been done to study the effect of polyploidy on self-incompatibility in *T. pratense.*

The effect of polyploidy on incompatibility has been reviewed in detail by LEWIS (1947) and ATWOOD and BREWBAKER (1953). After working with the known genotypes of autotetraploids in *Oenothera organensis*, LEWIS stated the following three principles which explain the results in most species that have been adequately tested.

(1) Some pairs of different *S* alleles compete in diploid pollen grains. The interaction results in compatibility of the pollen grain in a style carrying both the alleles.

(2) Pairs of *S* alleles which do not show competition may show dominance of one allele over another in the pollen grain.

(3) These two effects differ according to the species in their power to alter the incompatibility reaction.

Recently, the observations on the induced autopolyploids of *Trifolium repens* (ATWOOD and BREWBAKER 1953) and *T. hybridum* (BREWBAKER 1953) have been adequately explained on the above principles.

As most of the previous work has only dealt with the allelic interaction in the diploid pollen, other effects of polyploidy on the incompatibility cannot be ruled out. The present experiments on the tetraploid *Trifolium pratense* were taken up to find out whether the same principles could be applied to explain the results obtained in this species, and also to discover any new effects of polyploidy on the incompatibility mechanism. Such a study would seem to be a prerequisite for an efficient breeding programme with autoploids in clovers.

MATERIAL

Seeds from two crosses of unrelated plants in *T. pratense* L. were obtained from the Welsh Plant Breeding Station, Aberystwyth in 1951. The two families derived from them each gave four intra-sterile inter-fertile groups. Owing to the large number of *S* alleles found in the natural populations (BATEMAN 1947; WILLIAMS and WILLIAMS 1947) it was believed that the eight alleles involved in the two crosses were different. The entirely different behaviour of the *S* genotypes in the two families later supported this assumption.

TECHNIQUE

Induction of polyploidy

Autotetraploids were produced by means of colchicine. Solutions of 0.1, 0.2 and 0.4 percent were applied with or without the help of a small piece of cotton wool on

the growing points of the young seedlings at the rate of three drops a day for two days. A 0.2 percent solution used with cotton wool gave the best results.

Pollen tube studies

Pollen tube studies were made on the cut inflorescences in a dark incubator at 27°C. The styles were fixed in 3:1 acetic-alcohol for two hours and changed to 30 percent acetic acid for half-an-hour for softening. The styles were then transferred to Acid-Fuchsin-Light Green stain (DARLINGTON and LA COUR 1950) and were either put in a water bath at 60°C for half-an-hour or kept overnight before pressing between two slides for examination.

Testing the genotypes of tetraploids

The incompatibility behaviour in tetraploids cannot be understood unless the exact *S* constitution of the plants is known. When a normal diploid of known *S* alleles is doubled, we know that the tetraploids will contain two alleles of each type. But in the sexual progeny of two tetraploids, it is necessary to be able to discriminate between different genotypes. This is done by putting the pollen from diploid plants of known *S* constitution onto the stigma of the tetraploid. Three diploid plants of homozygous *S* constitution (S_1, S_2 and S_3) were available from X-ray induced 'revertible' mutations in the pollen. For the fourth allele (S_4) used in these experiments a suitable heterozygous diploid was used. Haploid pollen containing an *S* allele is inhibited in a tetraploid style carrying the same allele but any other pollen grows normally. This is readily ascertained in *T. pratense*, by the closing of the keel and the standard which accompanies fertilization.

Some examples of the testing method are shown as follows:

Compatible . . . +; Incompatible . . . −

Tetraploid females	Diploid male test plants					
	$S_{1.1}$	$S_{2.2}$	$S_{3.3}$	$S_{1.4}$	$S_{2.4}$	$S_{3.4}$
$S_{1.2(1.1)(1.3)(2.2)}$	−	−	+	+		+
$S_{2.3.4(2)(3)(4)}$	+	−	−		−	−
$S_{1.2.3.4}$	−	−	−	−		−

It is, however, impossible to determine by this method alone, to which of the three categories the diallelics belong. Plants showing the reaction $S_{1.2}$ may be $S_{1.1.2.2}$, $S_{1.1.1.2}$ or $S_{1.2.2.2}$, expressed collectively as $S_{1.2(1.1)(1.2)(2.2)}$ etc. Similarly in tri-allelics, it is impossible to distinguish which allele has been duplicated and this uncertainty is shown as $S_{2.3.4(2)(3)(4)}$ etc.

Sometimes the constitution of the di-allelics and tri-allelics may be inferred from the constitution of the parents. For instance, in the F_1 progeny of the two di-allelic tetraploid plants with no allele in common, *viz.* $S_{1.1.4.4} \times S_{2.2.3.3}$, a di-allelic plant $S_{1.3(1.1)(1.3)(3.3)}$ could only be $S_{1.1.3.3}$ and a tri-allelic plant $S_{1.2.3(1)(2)(3)}$ must be $S_{1.1.2.3}$. Thus in this type of cross, the complete genotypes of all the progeny can be determined.

TABLE 1

The number of genotypes found by analysing the progeny of two families, $S_{1.1.4.4} \times S_{2.2.3.3}$ *and the reciprocal, by pollinating each plant with four different diploid test plants. The expected values have been calculated on the basis of random chromosome segregation*

Genotypes	Observed no. of plants			Expected on the basis of 1:1:1:1:4:4:4:4:16
	$S_{1.1.4.4} \times S_{2.2.3.3}$	$S_{2.2.3.3} \times S_{1.1.4.4}$	Total	
$S_{1.1.2.2}$	0	1	1	0.805
$S_{1.1.3.3}$	0	2	2	0.805
$S_{2.2.4.4}$	0	0	0	0.805
$S_{3.3.4.4}$	1	0	1	0.805
$S_{1.1.2.3}$	2	0	2	3.222
$S_{1.2.2.4}$	2	3	5	3.222
$S_{1.3.3.4}$	3	0	3	3.222
$S_{2.3.4.4}$	2	1	3	3.222
$S_{1.2.3.4}$	9	3	12	12.888
Total	19	10	29	28.996

$\chi^2 = 4.185$ $\quad r = 0.8$ approx.

Tetraploid crosses

LEWIS (1947) suggested the terms 'balanced' and 'unbalanced' for the different tetraploid crosses according to the allelic constitution of the style and the pollen involved. They are 'balanced' when the relationship of the alleles in the pollen and the style is similar to either the incompatible or the compatible matings in diploids. 'Balanced' crosses comprise two groups: (1) (Balanced −) when both alleles present in the pollen are also present in the style, similar to incompatible crosses. (2) (Balanced +) when at least some pollen contains alleles (like or unlike) both of which are absent from the style, similar to compatible crosses.

The results of analysing the genotypes of two families raised from 'balanced +' crosses between $S_{1.1.4.4}$ and $S_{2.2.3.3}$ plants are given with numbers to be expected on random chromosome segregation in table 1. The results are in good agreement with expectation.

ALLELIC COMPETITION WITH SELF-POLLINATION

Pollen tube growth

All plants were self-pollinated and examined for pollen tube growth and seed production. Pollen tube measurements are given in table 2.

According to the pollen tube measurements, the genotypes could be grouped into three classes: (1) genotypes with short pollen tubes (6 to 7 units, 1 unit = 0.83 mm) comparable with the selfed diploid, (2) genotypes with long tubes (9 to 10.5 units), and (3) genotypes with long tubes reaching nearly to the ovary (10.6 to 12 units). In the di-allelics all the three classes are represented, in the tri-allelics only (2) and (3), and in the tetra-allelic, (3). This suggests a relationship between the genotype and the pollen tube growth.

In table 2 it is clear from the di-allelic genotypes $S_{1.1.2.2}$, $S_{1.1.4.4}$ and $S_{3.3.4.4}$ that

TABLE 2

Length of pollen tubes in selfed tetraploids after 24 hours at 27°C (3 to 5 styles measured in each plant)

Genotypes	No. of plants	Average length of pollen tube in units (1 unit = 0.83 mm)	Pollen genotypes	
			Homogenic	Heterogenic
Di-allelics				
$S_{1.1.2.2}$	2	5.3 ± 0.43	$S_{1.1}$, $S_{2.2}$	$S_{1.2}$
$S_{1.1.4.4}$	1	6.9 ± 0.82	$S_{1.1}$, $S_{4.4}$	$S_{1.4}$
$S_{1.1.3.3}$	1	11.4 ± 0.60	$S_{1.1}$, $S_{3.3}$	$S_{1.3}$
$S_{2.2.3.3}$	1	11.7 ± 0.20	$S_{2.2}$, $S_{3.3}$	$S_{2.3}$
$S_{2.2.4.4}$	1	9.9 ± 2.12	$S_{2.2}$, $S_{4.4}$	$S_{2.4}$
$S_{3.3.4.4}$	1	6.8 ± 0.27	$S_{3.3}$, $S_{4.4}$	$S_{3.4}$
Tri-allelics				
$S_{1.1.2.3}$	4	11.1 ± 0.42	$S_{1.1}$	$S_{1.2}$, $S_{1.3}$, $S_{2.3}$
$S_{1.2.3.3}$	2	11.2 ± 0.40	$S_{3.3}$	$S_{1.2}$, $S_{1.3}$, $S_{2.3}$
$S_{1.2.2.3}$	4	11.5 ± 0.44	$S_{2.2}$	$S_{1.2}$, $S_{1.3}$, $S_{2.3}$
$S_{1.2.2.4}$	5	10.3 ± 0.73	$S_{2.2}$	$S_{1.2}$, $S_{1.4}$, $S_{2.4}$
$S_{1.3.3.4}$	3	10.6 ± 0.62	$S_{3.3}$	$S_{1.3}$, $S_{1.4}$, $S_{3.4}$
$S_{2.3.4.4}$	2	11.0 ± 0.50	$S_{4.4}$	$S_{2.3}$, $S_{2.4}$, $S_{3.4}$
Tetra-allelic				
$S_{1.2.3.4}$	3	11.2 ± 0.44	—	$S_{1.2}$, $S_{1.3}$, $S_{1.4}$ $S_{2.3}$, $S_{2.4}$, $S_{3.4}$
Diploids				
Selfed (−)	4.0–6.6			
Crossed (+)	Bottom (12–13.5)			

pollen genotypes $S_{1.1}$, $S_{1.2}$, $S_{2.2}$, $S_{1.4}$, $S_{4.4}$, $S_{3.3}$ and $S_{3.4}$ produce only short pollen tubes. When the behaviour of these pollen types is known it is possible to infer the behaviour of the remaining pollen types. Thus, in the genotype $S_{2.2.4.4}$, it is pollen $S_{2.4}$ which gives rise to medium sized tubes. In $S_{1.1.3.3}$ and $S_{2.2.3.3}$ it is $S_{1.3}$ and $S_{2.3}$ respectively which produce long tubes. The one tri-allelic genotype, showing the medium tube, produces $S_{2.4}$ pollen and the others, showing long tubes, produce $S_{1.3}$ or $S_{2.3}$ or both types of pollen. The tetra-allelic genotype produces both types of long tubes, $S_{1.3}$ and $S_{2.3}$.

These results show that long or medium pollen tubes are produced only by heterogenic pollen. Not all of these heterogenic pollen types give rise to longer pollen tubes. It appears that some combinations of *S* alleles compete when together in the same pollen grain, so that neither allele can produce its normal effect while other combinations do not compete in this way.

In another family of *T. pratense*, the pollen tube growth in the induced tetraploids of the four *S* genotypes did not show any significant increase over that of the respective diploid genotypes.

(Pollen tube growth in units, diploid : tetraploid 4.5 ± 0.81 : 5.3 ± 0.07; 6.9 ± 0.38 : 9.1 ± 2.14; 4.9 ± 0.38 : 5.8 ± 0.41; 8.1 ± 2.50 : 8.1 ± 1.02).

Seed set

Diploid *Trifolium pratense* is highly self-incompatible and rarely produces any seed. Tetraploid plants behave according to the nature of the *S* alleles present. Table

TABLE 3

The seed setting ability of different di-allelic genotypes after selfing and compatible crossing. The origin of the tetraploid plants, whether they be the colchicine treated (c-induced) seedling or the progeny of the tetraploid crosses (T × T) is also mentioned

Genotype	Origin of the plant	No. of plants	Selfing			Compatible crossing
			No. of flowers	No. of seeds	No. of seeds per 10 flowers	No. of seeds per 10 flowers
$S_{1.1.2.2}$	T. × T.	2	36	0	0.0	3.7, 3.6
$S_{2.3.4.4}$	T. × T.	1	98	0	0.0	—
$S_{1.1.4.4}$	c-induced	3	577	0	0.0	5.0, 6.3
$S_{2.2.4.4}$	c-induced	1	429	1	0.02	—
$S_{1.1.3.3}$	c-induced	2	255	0	0.0	8.3
			373	0	0.0	5.5
	T. × T.	3	23	5	2.3	—
			52	6	1.2	—
			34	2	0.6	—
$S_{2.2.3.3}$	c-induced	1	144	19	1.3	7.2
	T. × T.	3	36	10	2.8	6.9
			89	22	2.5	7.0
			50	7	1.4	4.0

3 shows the seed producing ability of different di-allelic genotypes after selfing and compatible crossing. Among the six di-allelic genotypes obtainable from four different S alleles, three genotypes, $S_{1.1.2.2}$, $S_{1.1.4.4}$ and $S_{3.3.4.4}$ do not produce any seed and the genotype $S_{2.2.4.4}$ produced only one seed from 429 flowers selfed. As rare seeds are produced even in diploids, this genotype is also included with the other three. The two remaining genotypes $S_{1.1.3.3}$ and $S_{2.2.3.3}$ do produce some seeds, the $S_{2.2.3.3}$ generally more than $S_{1.1.3.3}$. But neither of these produces a full seed set determined by the seed set after compatible crossing, a comparison which largely excludes the effect of sterility due to polyploidy. These results are entirely consistent with the pollen tube measurements in these genotypes (table 2). Thus, though the incompatibility is greatly reduced in some allelic combinations, it is not enough to ensure regular fertilization. The pollen types $S_{2.3}$ and $S_{1.3}$ usually produce pollen tubes which nearly reach the ovary but the growth in the last few millimetres to the egg is still a matter of chance and not a certainty. This chance factor will be discussed when further data in this connexion have been considered.

There is a slight indication that the colchicine-induced C_1 generation of tetraploid plants set fewer seeds after selfing than plants obtained from tetraploid crosses (table 3). This might be due to the frequent occurrence of sectorial chimaeras in the treated plants or some unknown factor.

DOMINANCE

'Unbalanced' crosses

In a cross like $S_{1.1.2.3} \times S_{1.3.3.4}$, the male parent produces two kinds of pollen, *i.e.* (1) pollen, both alleles of which are present in the female parent, like $S_{1.3}$ and $S_{3.3}$, and (2) pollen having one allele which is present and the other not present in the

TABLE 4

The results of 'unbalanced' crosses, where the pollen genotypes that are likely to function, have one S *allele that is present and one that is not present in the female plant. Crosses in classes 1 and 5 are compatible and those in classes 2, 3 and 4 are incompatible. The compatible cross* $S_{2.3.4.4} \times S_{1.2.3.4}$ *produces very low seed set as the proportion of the functioning pollen grain,* $S_{1.3}$ *is only 16.6 percent of the total pollen produced. To denote the allelic constitution of the female parent in the cross, pollen grains are represented as* $S_{1.3}$ *when* S_1 *allele is present in either single or double dose and the* S_2 *not present in the style*

	Genotype	No. of flowers	No. of seeds	Seeds per 10 flowers	Unbalanced pollen genotypes in crosses	
					Incompatible	Compatible
Class 1	$S_{2.3.4.4} \times S_{1.3.3.4}$	19	8	4.2		$S_{1.\dot{3}}$, $S_{1.\dot{4}}$
	$S_{2.3.4.4} \times S_{1.2(2.3)(3.3)}$	19	4	2.1		$S_{1.\dot{3}}$, $S_{1.\dot{3}}$
	$S_{2.2.3.3} \times S_{1.2(2.3)(3.3)}$	23	14	6.1		$S_{1.\dot{2}}$, $S_{1.\dot{3}}$
	$S_{2.3.4.4} \times S_{1.2.3.4}$	16	1	0.6		$S_{1.\dot{2}}$, $S_{1.\dot{3}}$, $S_{1.\dot{4}}$
Class 2	$S_{2.3.4.4} \times S_{1.2.2.4}$	30	1	0.3	$S_{1.\dot{2}}$, $S_{1.\dot{4}}$	
Class 3	$S_{1.2.2.4} \times S_{2.3.4.4}$	17	0	0.0	$S\dot{2}_{.3}$, $S\dot{2}_{.4}$	
Class 4	$S_{1.1.2.2} \times S_{1.2(2.3)(3.3)}$	22	0	0.0	$S\dot{1}_{.3}$, $S\dot{2}_{.3}$	
	$S_{1.2.2.4} \times S_{1.2.3.4}$	24	0	0.0	$S\dot{1}_{.3}$, $S\dot{2}_{.3}$, $S_{3.4}$	
Class 5	$S_{1.1.2.3} \times S_{1.3.3.4}$	20	18	9.0		$S\dot{1}_{.4}$, $S\dot{3}_{.4}$
	$S_{1.3(1.3)(3.3)} \times S_{1.3.3.4}$	20	9	4.5		$S\dot{1}_{.4}$, $S\dot{3}_{.4}$
	$S_{1.2.2.3} \times S_{1.3.3.4}$	21	14	6.7		$S\dot{1}_{.4}$, $S\dot{3}_{.4}$, $S\dot{3}_{.4}$
	$S_{1.1.2.3} \times S_{1.2.2.4}$	24	19	7.9		$S\dot{1}_{.4}$, $S\dot{3}_{.4}$
	$S_{1.1.2.2} \times S_{1.2.3.4}$	15	8	5.3		$S\dot{1}_{.4}$, $S\dot{2}_{.4}$
	$S_{1.3.3.4} \times S_{1.2.3.4}$	16	12	7.5		$S\dot{1}_{.2}$, $S_{2.\dot{3}}$
	$S_{1.3.3.4} \times S_{2.3.3.4}$	15	3	2.0		$S_{2.\dot{3}}$, $S_{2.\dot{4}}$

female parent, like $S_{1.4}$ and $S_{3.4}$. It is the second type of pollen grain *i.e.* $S_{1.4}$ and $S_{3.4}$—having at least one allele that is not present in the female parent—which has any chance of being functional. The allele common to both pollen grains and the style may be present in single *e.g.* S_3 or double dose *e.g.* S_1 in the female parent. These differences in pollen grains have been denoted as $S\dot{1}_{.3}$ when S_1 is present in single or double dose and S_3 not present in the style. A number of crosses were made giving different combinations of S alleles in the 'unbalanced' pollen genotypes of the male parent. This presented an opportunity to study the dominance between the S alleles in the pollen grains.

Table 4 shows the results of 'unbalanced' crosses. Some of these crosses give full seed-set, *i.e.* 2–9 seeds per 10 flowers pollinated while others are sterile setting 0–0.3 seeds per ten flowers. However, there is one cross, $S_{2.3.4.4} \times S_{1.2.3.4}$ which is compatible but sets only 0.6 seeds per ten flowers, presumably because the proportion of the functioning pollen grain $S_{1.\dot{3}}$ is very meagre (16.6 percent).

Crosses in class 1 are fully compatible showing that in these crosses one or all of the 'unbalanced' pollen genotypes function. The functioning pollen genotypes can be identified by eliminating those pollen genotypes which are involved in incompatible crosses. Thus the pollen genotypes $S_{1.\dot{2}}$ and $S_{1.\dot{4}}$ which appear in the incompatible cross in class 2 are non-functional, leaving in class 1 only the pollen genotype $S_{1.\dot{3}}$ which must be compatible.

The classes 1 and 4 show that pollen $S_{1.3}$ is compatible whether S_3 is in single or double dose in the style but is incompatible when S_1 is present and S_3 is absent from

the style. This clearly indicates that the dosage difference in the style is of no consequence in the pollen and style reaction whereas the presence or absence of the particular allele in the pollen or style does seem to govern the compatibility or otherwise of the pollen.

Dominance in the pollen grain

In the 'unbalanced' crosses some types of pollen grains behave as if they contain only one allele, the other allele showing no reaction, *i.e.* one allele shows dominance over the other. If the *S* allele common to the style as well as pollen grain is the dominant one, the pollen is incompatible but if the other *S* allele in the pollen which is not present in the style is dominant, the pollen is compatible. It has been shown in table 4 that $S_{1.3}$ pollen grains are compatible in a style carrying S_2 but lacking S_1, and the same pollen grain on a style carrying S_1 but lacking S_3 is incompatible. This shows that S_1 is dominant over S_3 in the pollen grain. This is also suggested by the results in the progeny of the cross shown in table 5.

The data agree with the assumption that S_1 and S_2 are dominant over S_3 in the pollen.

The crosses given in table 4 are not sufficient to show directly the presence or absence of dominance in all the combinations of *S* alleles in the diploid pollen. For instance it is difficult to tell which of the pollen genotypes in class 5 are compatible.

TABLE 5

Genotypes of progeny in two families. The observed and expected values fit better with the assumption that S_1 *and* S_2 *are dominant over* S_3

Family T5/32		$S_{2.2.3.3} \times S_{1.1.3.3}$	
Genotypes	Observed	When S_1 is dominant over S_3	When S_3 is dominant over S_1 or there is no dominance
		Expected on the basis of 24:5:1 ratio	Expected on the basis of 4:1:1 ratio
$S_{1.2.3(1)(2)(3)}$	6	7.92	6.64
$S_{1.3(1.3)(2.3)}$	4	1.65	1.66
$S_{1.1.3.2}$	0	0.33	1.66
		χ^2 $P = 0.20 - 0.10$	χ^2 $P = 0.10 - 0.05$

Family T9/32		$S_{1.1.3.3} \times S_{2.2.3.3}$	
Genotypes	Observed	When S_2 is dominant over S_3	When S_3 is dominant over S_2 or there is no dominance
		Expected on the basis of 24:5:1 ratio	Expected on the basis of 4:1:1 ratio
$S_{1.2.3(1)(2)(3)}$	14	16.80	14
$S_{2.3(2.3)(3.3)}$	6	3.50	3.5
$S_{1.1.3.2}$	1	0.70	3.5
		χ^2 $P = 0.50 - 0.30$	χ^2 $P = 0.20 - 0.10$

However, the amount of seed set in different crosses provides a good clue in this direction.

From the crosses in the classes 2, 3 and 4 it is evident that either S_4 is dominant over S_1 and S_3 or there is no reaction between S_4 and any one of the alleles S_1 and S_3, when present together in the pollen. In the compatible cross $S_{2.3.4.4} \times S_{1.2.3.4}$ the seed set is very low. In this cross only $S_{1.3}$ pollen is compatible and so only 16.7 percent, (one sixth) of the total pollen produced by the male plant, functions. In the cross $S_{1.1.2.3} \times S_{1.3.3.4}$ where the seed set is very high, it seems that both the genotypes, $S_{1.4}$ and $S_{3.4}$, function, *i.e.* S_4 is dominant over S_1 and S_3. Similarly, from the cross $S_{1.1.2.3} \times S_{1.2.2.4}$ it may be inferred that S_4 is dominant over S_1 and S_2. In the cross $S_{1.3.3.4} \times S_{1.2.3.4}$ high seed set indicates that both the genotypes $S_{1.2}$ and $S_{2.3}$ function, showing that S_2 is dominant over S_1 and S_3. The low seed set in the cross $S_{1.3.3.4} \times S_{2.3.3.4}$ suggests that only $S_{2.3}$ pollen functions, and the other pollen type $S_{2.4}$ does not function because S_4 is dominant over S_2.

Thus S_4 is dominant over S_1, S_2 and S_3; S_2 is dominant over S_1 and S_3; and S_1 is dominant over S_3.

The effect of competition on pollen tube growth and seed set is shown in figure 1. Figure 2 shows the six pollen genotypes in the order of the strength of competition. Linear expressions of allelic competition and dominance have been shown in figures 3 and 4 respectively. The linear relationships of the two reactions, competition and dominance, are similar with regard to the allele S_4 and S_2 but the order of S_1 and S_3 is reversed. This indicates the relative independence of these two reactions. These interactions in the different genotypes are summarized below:

Interaction	Pollen genotypes					
	$S_{1.2}$	$S_{1.3}$	$S_{1.4}$	$S_{2.3}$	$S_{2.4}$	$S_{3.4}$
Competition	−	+	−	+	+	−
Dominance	S_2	S_1	S_4	S_2	S_4	S_4

S_4 is the strongest dominator but a very weak competitor, and S_3 is the weakest dominator but strongest competitor.

VARIABILITY IN THE INCOMPATIBILITY REACTION

Effects of temperature

After selfing, pollen tube growth was recorded in some diploid and tetraploid genotypes at three constant temperatures of 15°C, 20°C and 25°C.

In diploids the incompatibility reaction and normal compatible pollen tube growth behave similarly with a rise in temperature, both showing an increase. However, the optimum temperature for the maximum reaction differs in the two cases. For the incompatible reaction it is 20°C (fig. 5), but for the normal compatible pollen tube growth it may be 20°C or between 20° to 25°C or more (fig. 6). This shows that in the diploid, compatible pollinations are more sensitive to temperature than the incompatible pollinations.

Polyploidy greatly alters the threshold of the incompatibility reaction. The

COMPETITION & DOMINANCE RELATIONSHIP BETWEEN ALLELES

FIG. 1 COMPETITION, POLLEN TUBE GROWTH & SEED-SET

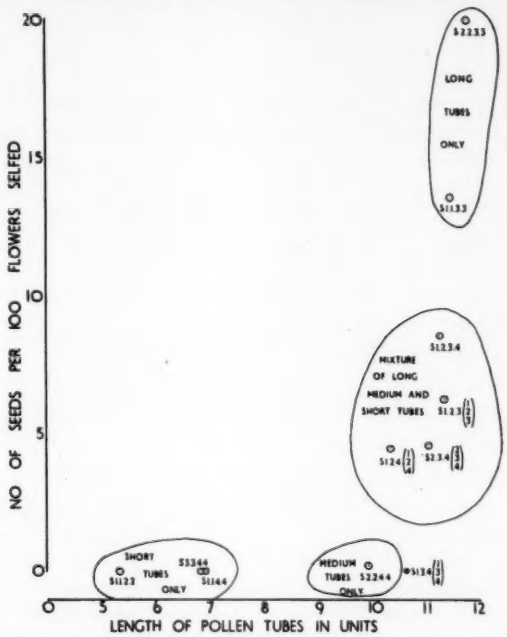

FIG. 2 ORDER OF COMPETITION IN 6 POLLEN GENOTYPES

STRONG COMPETITION						NO COMPETITION
S23	S13 S24		S14 S34		S12	
	STRONG		WEAK			

FIG. 3 LINEAR EXPRESSION OF ALLELIC COMPETITION

DIRECTLY CONNECTED ALLELES COMPETE
OTHERS DO NOT.

FIG. 4 LINEAR EXPRESSION OF ALLELIC DOMINANCE

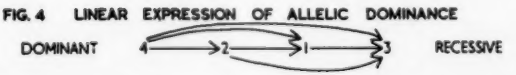

FIGURE 1.—Showing the relationship between allelic competition, pollen tube growth and selfed seed-set.

FIGURE 2.—Showing the different pollen genotypes in the order of their strength of competition reaction.

FIGURE 3.—Linear expression of allelic competition.

FIGURE 4.—Linear expression of allelic dominance.

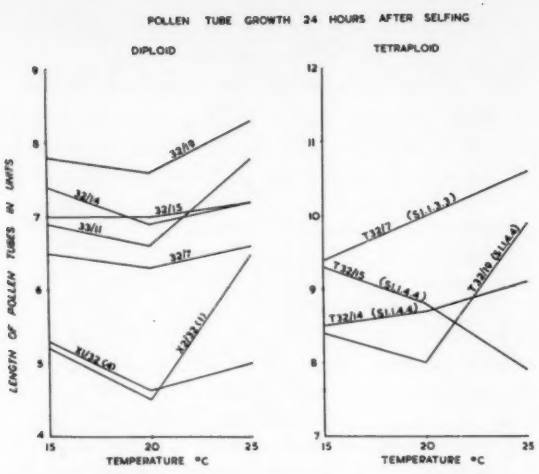

FIGURE 5.—Left. In diploids, the optimum temperature for incompatibility reaction is 20°C. Right. In tetraploids, the threshold of the incompatibility reaction is changed by polyploidy. Three plants belonging to the same genotype ($S_{1\text{-}1\text{-}4\text{-}4}$) show different reaction to increase in temperature.

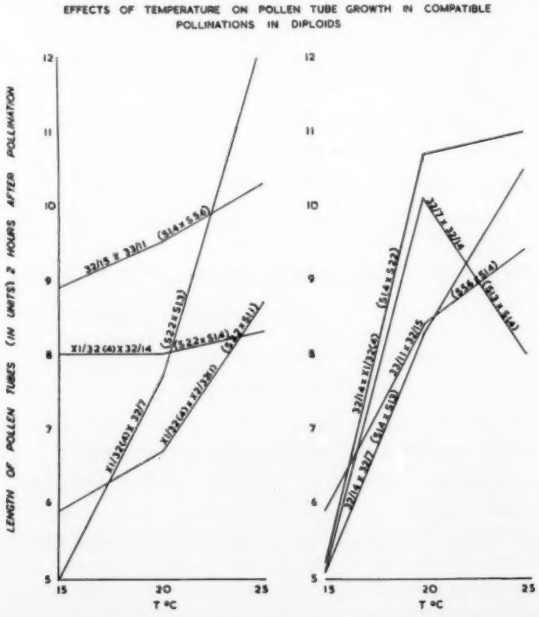
EFFECTS OF TEMPERATURE ON POLLEN TUBE GROWTH IN COMPATIBLE POLLINATIONS IN DIPLOIDS
LENGTH OF POLLEN TUBES (IN UNITS) 2 HOURS AFTER POLLINATION
T °C
T °C

FIGURE 6.—In compatible pollinations in the diploid, the reaction of pollen tube growth to temperature varies between different plants. Left. The increase in the rate of pollen tube growth is more between 20° and 25°C than that between 15° and 20°C. Right. The increase in the rate of pollen tube growth is more between 15° and 20°C than that between 20° and 25°C.

incompatibility reaction varies between different tetraploid genotypes as previously shown in table 2, but also varies a great deal within a genotype (fig. 5). In the genotype $S_{1.1.3.3}$ the response to the temperature is similar to that of normal compatible pollen tubes which show a steady increase in the growth rate with a rise in temperature. This means that the threshold has altered to such an extent that the direction of the reaction has been reversed from incompatibility to normal compatibility.

In the genotype $S_{1.1.4.4}$ the threshold of the reaction varies greatly within the genotype. This is reflected in the behaviour of the selfed pollen tube growth at different temperatures in the three plants, $T_{32/14}$, $T_{32/15}$ and $T_{32/19}$ belonging to this genotype. In $T_{32/14}$, the rate of pollen tube growth goes on increasing slightly as the temperature rises, indicating that the direction of the reaction may have been reversed from incompatibility to normal compatibility but the effect is too small to have a significant effect upon the pollen tube growth. In $T_{32/15}$, though the pollen tube growth is more than the other two at 15°C, it is strongly inhibited as the temperature rises. In this case the reaction is still towards incompatibility but the optimum temperature for the incompatibility reaction is nearer 25°C than 20°C which is typical of the diploids. In $T_{32/19}$, the threshold has not changed much for, as in the diploid, the incompatibility is strongest at 20°C but weaker at 15°C and 25°C.

Selfing and incompatible crosses

As expected from the results of self-pollination the crosses in the 'balanced −' group, where all the alleles present in the pollen parent are also present in the female parent, produce few seeds. Nevertheless a comparison of selfs and 'balanced −' crosses reveals a significant difference between them (table 6): selfing gives consistently higher seed setting than 'balanced −' (incompatible) crosses. The statistical analysis shows the difference to be highly significant at the one percent level.

Compatible crosses

In contrast to crosses of the 'balanced −' group, those of the 'balanced +' group produce at least one type of pollen containing two alleles, neither of which is present

TABLE 6

Results of selfing and incompatible crossing ('Balanced −') in tetraploids. Selfing gives considerably more seed set than incompatible crossing

Genotype	Crossing				Selfing			
	No. of crosses	No. of flowers	No. of seeds	Seeds per 10 flowers	No. of plants selfed	No. of flowers	No. of seeds	Seeds per 10 flowers
$S_{1.3(1.1)(1.3)} \times S_{1.3(1.1)(1.3)}$	3	68	2	0.3	3	79	7	0.9
$S_{2.3(2.2)(2.3)} \times S_{2.3(2.2)(2.3)}$	3	58	6	1.0	4	158	34	2.2
$S_{1.2.3(1)(2)(3)} \times S_{1.2.3(1)(2)(3)}$	5	156	0	0.0	4	160	15	0.9
$S_{1.2.3.4} \times S_{1.2.3.4}$	3	84	2	0.2	3	226	24	1.1
	Mean			0.4 ± 0.43	Mean			1.3 ± 0.62
	$t = 5.841$ at 1% level.							

TABLE 7

Results of group 'Balanced +' crosses, where each male parent produces at least one type of pollen containing two alleles, neither of which is present in the female parent of the cross. As expected, the seed set is high

Genotype	No. of crosses	Total no. of flowers	Total no. of seeds	Seeds per 10 flowers
Di-allelic × di-allelic				
$S_{1.1.4.4} \times S_{2.2.3.3}$	2	60	34	5.7
$S_{2.2.3.3} \times S_{1.1.4.4}$	1	19	14	7.4
$S_{1.1.3.3} \times S_{2.2.3.3}$	2	44	31	7.0
$S_{2.2.2.3} \times S_{1.1.3.3}$	1	20	14	7.0
$S_{1.1.2.3} \times S_{1.3.3.3}$	1	20	8	4.0
$S_{1.1.3.2} \times S_{2.2.3.3}$	1	21	7	3.3
$S_{2.2.3.3} \times S_{1.1.2.2}$	2	25	14	5.6
$S_{1.3.3.3} \times S_{1.1.2.2}$	1	21	11	5.2
$S_{1.3.2.3} \times S_{2.2.3.3}$	1	24	16	6.7
Di-allelic × tri-allelic				
$S_{1.1.2.3} \times S_{2.3.4.4}$	1	20	6	3.0
$S_{2.3.3.3} \times S_{1.2.2.4}$	1	19	17	8.9
$S_{2.2.3.3} \times S_{2\ 3.4.4}$	1	10	7	7.0
$S_{1.3.3.3} \times S_{1.2.2.4}$	1	19	10	5.3
$S_{1.3.3.3} \times S_{2.3.3.4}$	1	20	11	5.5
Di-allelic × tetra-allelic				
$S_{2.2.3.3} \times S_{1.2.3.4}$	1	20	12	6.0
$S_{1.3.3.3} \times S_{1.2.3.4}$	1	22	21	9.5
Tri-allelic × di-allelic				
$S_{2.3.4.4} \times S_{1.1.3.3}$	1	18	7	3.9
$S_{1.3.3.4} \times S_{1.1.2.2}$	1	25	13	5.2
$S_{1.3.3.4} \times S_{2.2.3.3}$	1	24	19	7.9
$S_{1.2.2.4} \times S_{2.2.3.3}$	1	24	10	4.2
$S_{1.2.2.4} \times S_{1.3.3.3}$	1	25	14	5.6
Tri-allelic × tri-allelic				
$S_{1.3.3.4} \times S_{1.2.2.4}$	1	16	10	6.3
$S_{1.1.2.3} \times S_{2.3.4.4}$	1	18	15	8.3
$S_{1.2.2.4} \times S_{1.3.3.4}$	1	24	6	2.5

in the female parent of the cross. In these crosses the seed set is high in accordance with expectation (table 7).

There is a significant difference in the seed set in the crosses according to whether only homogenic or heterogenic pollen functions. The average seed set per ten flowers for homogenic pollen is 4.8 ± 1.13, but for heterogenic pollen it is as high as 7.1 ± 1.72. This suggests that in *T. pratense* compatible heterogenic pollen was more effective in achieving fertilization than the homogenic pollen. The reason for this difference in efficiency is unknown.

DISCUSSION

Interaction between S alleles in the diploid pollen

Competition

Out of 12 pollen genotypes studied in *T. pratense*, only two showed enough competition to give some seeds after selfing. In *T. hybridum*, self-compatibility was obtained

only in two out of 39 genotypes studied (BREWBAKER 1953). Similarly in *T. repens* self-compatible genotypes were obtained only occasionally, in only one out of six genotypes studied by ATWOOD and BREWBAKER (1953). In *Oenothera organensis* competitive reaction was never strong enough to give any seed after selfing (LEWIS 1947).

These observations suggest that competition is infrequent in artificial autotetraploid. Interaction (whether competition or dominance) in the pollen must be a matter of chance, for there has been no direct selection for this character in the diploid. It is expected, therefore, that, while artificially induced tetraploids may exhibit some competition in the pollen, naturally occurring old tetraploids may not show any such reaction due to long term selective advantage for self-incompatibility.

Dominance

In contrast to the competition reaction, dominance is very common in all the three species of Trifolium. In Oenothera also, dominance is much more frequent and more effective than the competition reaction, though absence of dominance is also reported between some alleles. This is probably due to incomplete dominance rather than its absence. The preponderance of dominance in comparison with that of competition suggests that there may be some fundamental difference in the ways the two reactions—competition and dominance—are expressed in the pollen.

In competition reaction both the alleles in the pollen are active and work towards the same type of incompatible reaction. The breakdown of the incompatibility is probably due to the limited amount of the common substrate and the consequent lack of full expression of both the alleles in the pollen. In dominance only one allele is operating and the other is inert (recessive).

It is possible that a production of the inhibitory substance by the incompatible allele is generally stopped at the source by the compatible allele (cf. LEWIS 1947). Dominance, it appears, is a basic characteristic of *S* alleles and, like competition, has arisen in the absence of selective influence.

Interaction and plant breeding

In any breeding programme with species which are normally cross-pollinated, the degenerative effect of inbreeding has to be carefully considered. In breeding tetraploid *T. pratense* for a forage crop, the low seed setting is the major obstacle. Selection to improve fertility has been effectively carried on in Sweden for several generations. In this connexion, the self-compatibility due to polyploidy may become a great handicap for without care plants may be selected with alleles which show competition reaction. Selection of such plants will eventually give highly self-fertile types which may, sooner or later, degenerate due to inbreeding. It has been reported that over half of the plants in the Swedish alsike variety Tetra are self-compatible (BREWBAKER 1953). The original tetraploid stock as well as the plants selected in the first few generations should, therefore, be tested for self-compatibility so that self-fertile plants may be excluded. Fortunately the competition reaction is not a very common phenomenon in the three Trifolium species studied and this is probably also true of most of the other species showing oppositional type of self-incompatibility. Nevertheless, the selective advantage of the competing alleles may soon allow them

to spread widely in the succeeding generations of selection. However, competition interaction might prove to be of direct use in an inbreeding programme.

Dominance alone may have very little practical significance in any breeding programme or in changing the general fertility of a population; since with every combination of alleles that functions because of dominance, there is a reciprocal combination that fails to function.

Effects of modifiers

Pollen tube growth

In the diploid *T. pratense*, incompatible pollinations are less sensitive to temperature than the compatible pollinations. In Nicotiana incompatible pollen tube growth was remarkably constant in crosses involving similar *S* alleles, even though the sterility alleles were derived from different families which segregated for as many as six other genes (EAST 1934).

Polyploidy greatly alters the threshold of the incompatibility reaction. The threshold varies between different tetraploid genotypes but also varies a great deal within a genotype.

These facts suggest that the incompatibility reaction in diploids, after selfing or incompatible crossing, is generally so strong that minor modifying factors have no influence on pollen tube growth. However, in compatible crosses and in some tetraploid genotypes, where the incompatibility reaction is weakened due to interaction between the major *S* alleles, minor genes affecting the pollen tube growth are expressed and thus the sensitivity of the pollen tube growth to temperature and other external agencies is greatly increased.

Seed setting

Observations in *T. pratense* and also in *T. repens* (ATWOOD 1944; ATWOOD and BREWBAKER 1953) and Oenothera (LEWIS 1947) have shown that autotetraploids give higher seed set on selfing than on incompatible crossing.

A search for data on incompatible selfing and crossing in various species at the diploid level, *Prunus avium* (2x = 16)—CRANE and LAWRENCE (1931), CRANE and BROWN (1937); *Pyrus communis* (2x = 34)—CRANE and LEWIS (1942); *Trifolium pratense* (2x = 14)—WILLIAMS and WILLIAMS (1947); *Trifolium hybridum* (2n = 16)—WILLIAMS (1951), revealed that the seed set after selfing in the incompatible or pseudo-incompatible types is generally lower than that in the incompatible crosses. Sometimes selfing and crossing gave the same seed set but only rarely was a case found where selfing gave more seed set than crossing. This suggests that the technique of emasculation and crossing is not a cause of the lower seed set with incompatible crosses in the tetraploid.

The self-incompatible species are naturally cross-pollinated. This allows the diploid (as well as the tetraploid) to accumulate recessive lethal genes under the cover of heterozygosity. These plants are liable to show a marked effect of inbreeding depression. A very low percentage of self-fertilisation in these plants is thus further handicapped by homozygous lethals. In cross-incompatible pollinations, however, there is less inbreeding depression and most of the fertilisations will produce seed.

In the freshly induced autotetraploids, however, there will be a pair of each recessive lethal gene as well as the dominant viable genes. In this case the recessive lethals are more likely to be covered by the dominant viable genes, thus the seed set on selfing should be nearly equal to, but never more than, that of the incompatible crossing if other factors were not involved. In contrast, tetraploids show considerably higher seed set on selfing than on incompatible crossing. Some other factors must be operating.

In some tetraploid genotypes the incompatibility is greatly broken down to allow the pollen tubes to reach nearly to the ovary (*viz.* in pollen $S_{1.3}$ and $S_{2.3}$) but is still strong enough to check them from traversing the last few millimeters to the egg. In such cases a small variability due to minor genes in the pollen will be reflected in the pollen tube growth and will allow an occasional production of seed. This assumption will explain the small percentage of seed set after selfing in some plants only and not in others of the same genotype but will not explain why it should generally give more seed in selfing than in incompatible crossing.

This could be explained by the following assumptions:—

If these minor genes (1) are weak compatibility genes which (2) promote pollen tube growth if the same genes are present in the pollen and the style and (3) are ineffective in the pollen if not supported by the similar genes in the style.

With these assumptions the variability in the pollen grains due to the various combinations of the minor genes will be advantageous for seed setting only when selfed but will be ineffective in incompatible crosses.

These minor compatibility genes will have to develop in the face of the unfavourable selection which is definitely towards the strong incompatibility system. Nevertheless, if it is assumed that the accumulation of these minor compatibility genes took place comparatively late under the cover of the strong S alleles, this hypothesis is quite feasible.

SUMMARY

1. In *Trifolium pratense*, a highly self-incompatible species, reactions of S alleles and other minor genes controlling incompatibility have been studied in induced autotetraploids.

2. The S alleles in the diploid pollen genotypes $S_{1.3}$, $S_{2.3}$ and $S_{2.4}$ show allelic competition which results in long pollen tubes in styles with the same alleles, and selfed seed is produced in $S_{1.1.3.3}$ and $S_{2.2.3.3}$ plants. The other pollen genotypes, $S_{1.2}$, $S_{1.4}$ and $S_{3.4}$ produce short tubes and therefore do not show competition.

3. In some diploid pollen genotypes S alleles show dominance; S_4 is dominant over S_1, S_2 and S_3; S_2 is dominant over S_1 and S_3; and S_1 is dominant over S_3.

4. Unlike the diploids, different individuals of the same S genotype in tetraploids have different self pollen tube growth, different selfed seed set, and different reactions of their pollen tubes to temperature. It is suggested that the major weakening of incompatibility by allelic competition allows modifier genes, which have no obvious effect in the diploids, to manifest themselves.

5. In self-incompatible diploid species incompatible crossing gives more seed than on selfing. This is most probably due to inbreeding depression. In artificially induced

autotetraploids, however, selfing gives more seed set than incompatible crossing. This has been explained by assuming the presence of modifier genes.

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ISOLATION OF PRE-ADAPTIVE MUTANTS IN BACTERIA BY SIB SELECTION[1]

L. L. CAVALLI-SFORZA[2] AND JOSHUA LEDERBERG

Department of Genetics, University of Wisconsin, Madison, Wisconsin and Istituto Sieroterapico Milanese "Serafino Belfanti", Milan

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THE ROLE of antibiotics in provoking the development of resistant mutants of bacteria has been repeatedly discussed (CAVALLI 1952; Society of General Microbiology Symposium 1953; CAVALLI and LEDERBERG 1953; BRYSON and SZYBALSKI 1955; SEVAG, REID and REYNOLDS 1955). The two principal conceptions may be labelled the pre- and post-adaptation theories, respectively, according to whether the adaptive hereditary alteration occurs in individual cells prior to their contact with the drug, or in consequence of it. One basis of decision has been the isolation of resistant mutants by indirect (sib clone) selection (LEDERBERG and LEDERBERG 1952). The over-all result of this method is the isolation of lines of resistant mutants that have never been exposed to the drug in question, and are therefore indisputably preadaptive. This method, which depends on a technique of replica plating with a velvet sheet from one agar plate to another, gives a clear-cut qualitative result but is inherently not quantitative, so that failures are difficult to assess. In this paper, a more general procedure for indirect selection that allows of quantitative control will be described in its application to resistance to streptomycin and chloramphenicol, respectively, in *Escherichia coli*.

INDIRECT SELECTION IN LIQUID MEDIA: LIMIT SAMPLING

The principal difficulty in assessing mechanisms of drug resistance is the rarity of resistant cells. On the preadaptation theory, a population of bacteria may already contain, say one resistant per million cells. Short of individual tests on a million isolates, on one hand, and the direct but ambiguous use of the antibiotic as a selective agent on large populations, on the other, how can these rare resistant cells (or clones from them) be extracted? The method to be described depends on the indivisibility of single cells in progressively smaller samples of these populations.

Consider, for example, a population containing 10^9 sensitive and 10^3 resistant organisms per ml. A 0.1 ml sample will contain, on the average, 10^8 sensitives and 100 resistants. But a 10^{-4} ml sample will contain 10^5 sensitives and 0.1 resistant cells. What is 0.1 cell? The statement refers, of course, to the expectation that of ten samples, nine will contain no resistants, the tenth will contain one. In this tenth sample, however, the ratio of resistant to sensitive will have been raised from $1:10^6$ to $1:10^5$, a tenfold enrichment. If the resistant cells can hold their own in mixed

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[2] Fellow of the Rockefeller Foundation at the University of Wisconsin.

culture with sensitives, such a sample can be inoculated in fresh broth, and give rise to a new population with 10^9 sensitive and 10^4 resistants. The same enrichment procedure can then be reiterated until the resistant clone is obtained in pure culture, in six calculated cycles. At each stage, ten samples must be taken that have an expectation of 0.1 resistant cell each; the one which actually contains it is identified retrospectively by assays on samples of each culture. These assays further serve to guide the practice of the next cycle.

For a quantitative treatment, consider an initial culture with N_0 cells of which R_0 are resistant, giving a ratio $r_0 = R_0/N_0$. If samples of size q are taken they will contain $m = qR_0$ resistants, and $qN_0 = m/r_0$ total cells. If the sample is then distributed among n tubes of fresh medium, each tube will receive m/nr_0 cells, and, if $m \ll n$, either zero or one resistant. In the latter tubes, the proportion of resistant cells in the inoculum will be $1/\frac{m}{nr_0} = nr_0/m = r_1 = R_1/N_1$. If the ratio of resistant cells remains unaltered during growth, the final culture will have been enriched by a factor $E = r_1/r_0 = n/m$.

However, if the ratio is altered owing to differential growth, the resistant ordinarily being slower, the effective enrichment $E(t)$ will be a function of the amount by which the inoculum is permitted to grow. If we take t as the number of e-fold increases of the sensitive cells, k as the relative growth rate of the resistants, and $R_1 = 1$, the sensitives will grow as $(N_1 - 1)e^t$, and the resistants as e^{kt}. Thus $r_1(t) = R(t)/N(t)$ will be given by

$$\frac{1}{r_1(t)} = \frac{(N-1)e^t + e^{kt}}{e^{kt}} = \left(\frac{1}{Er_0} - 1\right) e^{t(1-k)} + 1 = \left(\frac{1}{Er_0} - 1\right) (R_1(t))^{1-k/k} + 1, \tag{1}$$

and approximately, if $Er_0 \ll 1$ and $r_1(t) \ll 1$,

$$E(t) = Ee^{t(k-1)} \tag{2}$$

which approaches zero asymptotically with increasing t. From (2) also, $E(t) \leq 1$ and enrichment becomes impoverishment after $t = \log E/(1 - k)$. It is therefore desirable, or even essential, to limit the number of generations as much as convenient.

In the above discussion, we have taken R_1 as either zero or one; actually both R_1 and $m = qR_0$ follow the Poisson series and may take any integral value. The sample with expectation m might contain no resistants at all with a probability $e^{-m} = e^{-qr_0N_0}$, so that none of the tubes would be "positive" (i.e., no tube will have had at least one resistant in the inoculum) and the enrichment cycle would fail entirely. For this reason, the positive tube of each cycle should be saved until the next cycle has been successfully completed.

The procedure may be formulated more generally as relying upon the calculated dispersion of r around its mean R_0/N_0 and the retrospective choice of those samples in which r had been the highest. When N_0 is large, its variation may be ignored,

TABLE 1

Distribution of resistant mutants in samples of 100 cultures. In addition to the numbers shown, 87 cultures had zero resistants

Tube no.	No. of S^r colonies
3	9
15	1
30	4
32	2
41	37
44	2
47	1
57	3
67	1
78	19
83	2
84	4
100	5

while in small samples R follows the Poisson series, so that a calculable fraction of samples will have a ratio R_0/N_0 which exceeds any stated value. The procedure is thus applicable, in principle, for any value of m but is most efficient for $m < n$.

The calculated enrichment E can also be adjusted to minimize the total number of tubes required to achieve complete enrichment, $R = N$, which will take i cycles where $E^i = 1/r_0$. The total number of tubes Ei will then be minimal for $E \sim e$ and $i \sim \log 1/r_0$. As a compromise between economy of time and of material, E was chosen around 10 in the experiments reported here.

As m, n can be established in advance, and k can be measured, it is possible to estimate with fair accuracy the enrichment expected and compare it with the enrichment observed; the agreement between the two values will supply a test that the resistant mutants are actually present in the right number in the culture untreated with the antibiotic, and thus that all of the resistant mutants recovered by direct assay with the drug are spontaneous mutants.

RESISTANCE TO STREPTOMYCIN

Escherichia coli W-1802 (a stock from the K-12 strain) was used for the experiment. A single-colony culture was used for the inoculation of 100 tubes containing 7 ml each of penassay broth, all tubes being seeded with about 100 cells. At the end of incubation half of each culture was plated on nutrient agar containing 100 μg/ml of streptomycin, while the remaining half was stored in the refrigerator.

The plates from 87 tubes showed no growth on streptomycin, while the counts of streptomycin resistant organisms from the remaining 13 tubes were as given in table 1.

The "best" culture (i.e., with maximum r_0) (No. 41) contained 37 resistants in the moiety which was assayed on streptomycin, and therefore about 10 resistant cells per ml. From the rest of culture 41, meanwhile kept at 4°C a sample of 0.1 ml, which was expected to contain one resistant organism, was mixed with 100 ml

of broth. From this dilution ten tubes were made, each containing 10 ml of broth, and incubated. To reduce the chance that the enrichment cycle would fail, another series of 10 tubes with 10 ml broth was prepared with an 0.2 ml sample from culture 41, thus expected to contain two resistant organisms.

After the 20 tubes were grown to saturation a sample was taken from each and plated on streptomycin agar. The estimates of resistant organisms per ml were as follows:

First enrichment from culture 41

Sample seeded	No. of resistant organisms per ml in each of the ten cultures									
0.1 ml	0	4	0	0	0	0	0	0	0	0
0.2 ml	0	0	**33**	9	11	**35**	0	0	6	0

It is difficult to judge whether the second culture of the first series and the fourth, fifth, ninth culture of the second series contain the same mutant as the original culture or new mutants. It is very likely instead that the third and sixth culture of the second series contain the same mutant as in the original, enriched 3.3 and 3.5 times respectively by the procedure. The expected enrichment for $k = 1$ is $5 \times$ in the second series, there being $n = 10$ tubes and $m = 2$ resistant organisms in the inoculum distributed into the ten tubes.

As a control, one of the original 100 cultures (No. 42) which showed no resistant organisms in the sample plated on streptomycin was treated in parallel to culture 41 and exactly in the same way. The results were:

Examination of culture 42 in parallel with culture 41

Sample seeded	No. of resistant organisms per ml in each of the ten cultures									
0.1 ml	0	0	0	0	0	0	0	0	0	2
0.2 ml	0	0	0	0	0	0	0	0	0	0

In the series from culture 42 no enrichment was expected, there being no resistant mutants to be enriched upon, and none is indeed found (except "background" mutants, e.g. in the tenth culture of the first series).

The total count in the conditions employed was 1.7×10^9 bacteria per ml. At the beginning there were in the best culture 10 resistants per ml, or a ratio $r_0 = 6 \times 10^{-9}$. After the first cycle of enrichment the concentration of resistants had increased to a maximum of 21×10^{-9} and the best culture was chosen for a further enrichment cycle. It was thus possible to reach, by a succession of fourteen cycles conducted in a way identical or very similar to the first one just described, a concentration of resistant organisms such that plating of the culture on nutrient agar and direct scoring for resistance of the colonies thus obtained permitted a line made entirely of streptomycin-resistant cells to be indirectly selected.

The details of the successive enrichments are given in table 2. It was soon clear, from the comparison of observed and expected enrichments, that the resistant mutant was multiplying more slowly than the sensitive parent. In order to lessen this obstacle from adverse selection the following means of reducing t, the number of multiplications were tried: diluting the broth, (1/10 or 1/100) and decreasing the

TABLE 2

Indirect selection for streptomycin-resistance. Summary of enrichment cycles

	Total conc. of cells/ml (1)	Broth		Max. resistant assay cells/ml (3)	Relat. concentration of resistants after cycle		Observed enrich-ment (6)	n (7)	m (8)	Expected enrich-ment n/m (9)	k (10)
		Amount (2)	Dil.		Max. (4)	Average (5)					
Original culture	1.7×10^9	7 ml	1/1	10	5.9×10^{-9}	—	—	—	—	—	—
Cycle 1	1.7×10^9	10 ml	1/1	35	2.1×10^{-8}	2.0×10^{-8}	3.40	10	2	5.0	.94
2	1.7×10^9	10 ml	1/1	58	3.4×10^{-8}	3.2×10^{-8}	1.52	10	2	5.0	.84
3	1.7×10^9	10 ml	1/1	140	8.2×10^{-8}	5.9×10^{-8}	1.74	13	2	6.5	.84
4	1.7×10^9	10 ml	1/1	456	2.7×10^{-7}	1.41×10^{-7}	1.72	20	1	20	.76
5	1.7×10^9	10 ml	1/1	1400	8.2×10^{-7}	7×10^{-7}	2.59	20	2	10	.88
6	3.1×10^9	10 ml	1/10	790	2.5×10^{-6}	2×10^{-6}	3.05	10	2	5	.95
7	1.05×10^8	10 ml	1/50	1250	1.2×10^{-5}	1.2×10^{-5}	4.80	10	2	5	.99
8	1.7×10^9	10 ml	1	1.53×10^4	0.9×10^{-5}	0.53×10^{-5}	0.44	20	2	10	.78
9	3.1×10^9	0.1 ml	1	1.34×10^5	4.3×10^{-5}	43×10^{-5}	4.78	20	2	10	.93
10	3.1×10^9	0.1 ml	1	3.1×10^5	1×10^{-4}	0.98×10^{-4}	2.28	23	2	11.5	.86
11	3.1×10^9	0.1 ml	1	7.0×10^5	2.3×10^{-4}	1.19×10^{-4}	1.19	20	3	6.7	.86
12	3.1×10^9	0.1 ml	1	4.7×10^6	1.5×10^{-3}	1.13×10^{-3}	4.91	20	2	10	.95
13	3.1×10^9	0.1 ml	1	7.8×10^6	2.5×10^{-3}	1.8×10^{-3}	1.20	20	2	10	.87
14	3.1×10^9	0.1 ml	1	3.96×10^7	1.28×10^{-2}	5.5×10^{-3}	2.20	40	4	10	.90

$k = .882 \pm .018$.

size of the culture. The latter method was found more satisfactory. Reconstruction experiments showed in fact that while dilution of the nutrient broth with saline did not decrease proportionately to dilution the final concentration of bacteria at saturation, the disadvantage of the resistant increased at lower broth concentration. Another simple device that could reduce the number of doublings in the fresh medium, and thus improve the enrichment if $k < 1$, might be that of stopping growth before saturation. However, it was preferred to use saturation as the endpoint of growth because it was already known that a saturated culture, stored at 4°C, kept the ratio of the resistant cells to the total unaltered for over a week. Such a stabilization for at least 24 hours and preferably several days is essential for this kind of experiment, because the enrichment tubes must be stored until they have been assayed for resistants and the next enrichment cycle started.

The comparison of the enrichment observed (col. 4, table 2) with that expected, $E = n/m$ for $k = 1$ (col. 6, table 2) permits one to calculate, by the use of formula (1), the value of k (the relative growth rate of the resistants) that would explain the observed enrichment rates. k was found to be $0.88 \pm .02$.

Recovery of the resistant line that was selected by indirect selection permitted the value of k thus calculated to be compared with that obtained directly from reconstruction experiments. Mixtures were made in various initial proportions of S^s and S^r cells, and the ratio between the two types of cells were measured at the end of growth. An average of two experiments gave $k = 0.856 \pm 0.013$, in good agreement with the value obtained from the indirect selection experiment. In reconstruction experiments with mixtures of sensitive and streptomycin resistant mutants of *E. coli*, CEPPELLINI (1954) had also obtained a closely similar value of k.

It will be noticed that, for enrichment, whenever more than one "good" tube was available, the best tube (i.e. the one showing the highest assay of resistants, given as Max. in col. 2 in table 2) is to be chosen. Both average and maximum concen-

trations of resistants after each cycle are given in column 3 of table 2. Observed enrichment (col. 4) is calculated as the ratio of the average relative concentration of resistant mutants after one cycle, and the concentration of resistants in the best tube before the cycle, the best tube having of course been employed for continuing selection.

Enrichment cycles were missed three times, i.e. no resistant cell showed up in the tubes after dilution. Thus, altogether seventeen cycles were made; the third, eighth and eleventh cycles had to be repeated. As already stated the probability of "missing" a cycle is $e^{-1} = 0.37$, if one resistant cell is seeded; $e^{-2} = 0.14$ if two resistant cells are seeded; $e^{-3} = 0.05$ if both series with 1 and 2 resistant cells are employed, as in most cycles of the present experiment. Failures were 3/17, i.e. slightly more frequent than expected, but not significantly so.

At the end of the fourteenth cycle, the relative concentration of streptomycin resistant cells was estimated to be 1.28% in the best tube as shown by the assay on streptomycin agar. The culture concerned was plated on nutrient agar (without streptomycin) and 149 colonies were subcultured on nutrient agar and tested individually for streptomycin resistance by planting them on agar with and without the drug. Two colonies (1.34%) were resistant.

One resistant isolate was restreaked on plain agar, then inoculated into penassay broth from which twenty serial passages of .001 ml in 10 ml were made in the course of one month. These passages entailed a cumulative total of about 260 generations. The twentieth passage culture was then plated out on agar with and without streptomycin, giving the following assays: on plain agar, $4.1 \pm .1 \times 10^9$; on agar with 200 μg/ml streptomycin, $4.3 \pm .1 \times 10^9$; on agar with 1000 μg/ml streptomycin, $4.2 \pm .2 \times 10^9$ per ml. In addition several hundred colonies on the plain agar plates were tested by replica plating to streptomycin agar without any sensitive reversions being detected. This indirectly selected resistant clone, which has never been exposed to streptomycin, may be concluded to be stably and completely resistant to streptomycin.

One more fact which was noticed in the present experiment is worth comment. When more than one tube of a series was "positive", a high variability of the number of resistant cells was almost always present among tubes. It must be remembered that the number of resistant cells in one of these tubes represents the total progeny of (usually) one resistant cell in a fixed time, i.e. the time necessary for the majority of the cells, the sensitive ones, to reach saturation.

This variability may reflect the variability in lag of individual cells and perhaps the variability in the time of individual divisions (KENDALL 1953). This variability is of such a size, that it may occasionally reverse enrichment into impoverishment, as has happened at the 8th cycle.

HERITABILITY OF VARIANCE OF STREPTOMYCIN RESISTANTS

Table 1 shows the variability in yields of resistant mutants from culture to culture, a variation that has been ascribed to preadaptive mutations by LURIA and DELBRÜCK (1943), but to physiological response to uncontrolled environmental variation by DEAN and HINSHELWOOD (1952). To test the heritability of this variation, 10

samples of one ml each were reinoculated into 7 ml fresh penassay broth, both from culture 41 (which had a calculated level of about 10 resistants per ml) and 42 (which had none). Reassays of the whole cultures gave the following results:

From culture 41: 135, 142, 181, 123, 177, 111, 138, 149, 140, 144

From culture 42: 0, 0, 8, 2, 0, 0, 0, 1, 0, 1

These results should be considered together with those of more dilute samples from the same cultures, page 370.

If the difference between the original culture 41 and 42 is to be ascribed to the physiological state of the cells, it would then be necessary to assume that this difference is heritable with the further growth of the cells in the absence of the drug.

INDIRECT SELECTION FOR CHLORAMPHENICOL RESISTANCE

Chloramphenicol resistance offers a particular challenge for various reasons. Resistant mutants are found which show only a moderate degree of resistance; only by the cumulation of independent mutations it is possible to reach high degrees of resistance (CAVALLI and MACCACARO 1952). Single resistance steps do not permit complete (i.e. 100%) survival at the concentrations of drug which are necessary for a complete inhibition of the sensitive parents. The increase of resistance in "training" experiments is therefore almost perfectly gradual, a trend particularly acceptable to advocates of the post-adaptation theory.

Moreover, the survivorship varies with the medium, time of incubation, amount of background of sensitives, and other conditions. In view of incomplete survival, it has to be assumed that assays on drug agar gave a variable underestimate of the number of resistant cells. As, for a rapid application of the enrichment method here employed, it is essential that limiting dilutions are used, five series of broth tubes (each of $n = 10$ tubes) were employed, containing respectively 2, 1, ½, ½, ⅛ resistant cells (as estimated from the assay). Thus, even if the assay estimate were only 12.5% of the true number of resistants, the last series would contain just one resistant. Actually, later tests, which became possible when the indirectly selected resistant line was secured, showed that the estimate from the assay may even be lower than 12.5%.

An *E. coli* stock (from the K-12 line) No. 373 was employed. A single colony culture was used for the inoculation of 0.5 ml broth cultures, 50 in all, 100 cells being seeded in each tube. After saturation, 0.1 ml from each culture was plated on a penassay agar plate containing 18 μg/ml chloramphenicol. The rest of each culture was stored in the refrigerator. Plates were incubated 48 hours, this time being necessary for the development of a good fraction of the colonies. At the time and concentration of cells and drug employed, resistant colonies were mostly of a pin-point size but there was no background growth, which would otherwise make counts difficult. Counts of resistant colonies were:

Chloramphenicol resistance, distribution of resistants

No. of resistant colonies	0	1	2	3–4	5–8	9–16	17–32	Tot.
No. of plates	17	14	4	5	6	3	1	50

TABLE 3

Number of chloramphenicol resistant organisms in subcultures from culture No. 40, containing originally 230 resistants/ml, from which samples were taken expected to contain 2, 1, ½, ¼, ⅛ resistant. Boldface indicates cultures which are believed to have been seeded with at least one resistant mutant

	Enrichment series				
Expected no. of positive cultures in each series	2	1	½	¼	⅛
Number of resistants in chloramphenicol agar plates prepared from each tube	16	15	1	0	0
	7	23	62	18	2
	7	15	***216***	0	5
	33	2	2	0	1
	9	2	1	0	25
	4	0	1	4	2
	16	49	9	7	0
	1	25	10	29	2
	27	1	1	1	0
	2	**131**	0	8	24

TABLE 4

Number chloramphenicol resistant organisms in subcultures from culture No. 39, containing originally no resistant mutant, treated exactly as culture No. 40, table 3

	Enrichment series				
Expected no. of positive cultures in each series	2	1	½	¼	⅛
Number of resistants in chloramphenicol agar plates prepared from each tube	2	39	7	14	2
	5	5	7	16	7
	2	32	1	10	6
	2	1	4	4	7
	13	18	2	19	0
	10	22	1	0	1
	1	17	5	1	0
	16	16	17	14	2
	4	47	3	18	7
	1	39	48	32	3

Culture No. 40 contained the highest number of resistants (23 in 0.1 ml, or r a little less than 10^{-7}).

From culture 40 a sample was taken and diluted so that samples of 5 ml of broth would contain each 2, 1, ½, ¼, ⅛ resistant cells. These numbers will thus represent also the approximate expectations for the numbers of positive cultures in each series. Culture No. 39 which gave no resistant colonies on the drug plate was treated in exactly the same way, as a control. The 5 ml samples were then distributed into ten small test tubes, each receiving thus 0.5 ml of broth. After incubation, 0.1 ml from each culture was spread on 18 μg/ml chloramphenicol agar, while the remaining 0.4 ml of each culture were stored in the refrigerator. Counts of resistant colonies after 48 hours on chloramphenicol agar were as given in tables 3 and 4.

In the first cycle of indirect selection it is often difficult to distinguish whether the resistants appearing on the plates are clonal descendants of those in the original

culture, or new mutants. Assuming that the resistant cells in the original culture (No. 40) were 23 in 0.1 ml of broth, in the same amount of broth after enrichment the following numbers of resistants would be expected in the five series inoculated with culture 40.

Resistants inoculated and expected number of positive cultures	2	1	½	¼	⅛
Enrichment ratio	5	10	20	40	80
Expected number of resistants after enrichment in 0.1 ml of positive cultures from culture 40, table 3.	115	230	460	520	1040

If, however, 23 is an underestimate of the resistant cells due to the difficulties of assay mentioned above, the expected numbers of resistants will be overestimated.

Taking 100 to be the maximum number of "new" resistants that may be counted in 0.1 ml, one finds that while no clear enrichments are observed in the first series (with 2 organisms), in the second series (with 1) one culture, and in the third (with ½) another culture (indicated by boldface, table 3) showing enrichment are available. The limit of 100 resistants is of course arbitrary, but has at least the advantage that it is not overtaken in the control series (culture 39, table 4) where no enrichment is expected.

Unless the mutant has a smaller growth rate than the sensitive, it may be argued from the figures observed that 23 was an underestimate, and therefore that the figures of enrichment expected in the calculation above are overestimated. If this is so, the numbers of resistants expected in the first series are not sufficiently high to permit the old clones to be distinguished from the background of new mutants, as indeed seems to have occurred.

Choosing the best of the enrichment cultures, i.e. the third culture of the third series for the second cycle, and diluting it again in broth so as to have 5 ml samples containing each 2, 1, ½, ¼, ⅛ resistant cells, and distributing these in 10 small tubes as before, subculture of them and plating on chloramphenicol agar gave the counts reported in table 5.

The following facts are worth noting: underestimation of resistants in the plate

TABLE 5

Second enrichment cycle, from the culture indicated in table 3 as 216

	Enrichment series				
Expected number of positive cultures	2	1	½	¼	⅛
Resistants in plates prepared from each of the ten cultures of the series	86	24	15	13	2
	38	26	3	2	0
	178	25	1	6	9
	8	**107**	0	1	5
	18	***814***	10	9	0
	215	7	**450**	0	2
	92	**208**	23	0	4
	248	**161**	4	1	0
	67	0	5	0	**246**
	42	0	3	10	**135**

TABLE 6

*Third enrichment cycle, from the culture indicated in table 5 as **814***

	Enrichment series				
Expected number of positive cultures	2	1	½	¼	⅛
No. resistants in plates prepared from each of the ten cultures of the series	**292**	**210**	2	0	0
	3	0	0	1	3
	63	0	7	6	0
	0	**360**	0	0	4
	2	18	**325**	***940***	2
	144	**164**	**790**	0	0
	340	0	0	9	0
	0	0	0	0	0
	0	0	18	**544**	0
	191	0	**273**	0	0

assay is more prominent than before; the variability of counts in parallel positive cultures is remarkably high; enrichments observed are therefore somewhat erratic, but clearly higher than in the previous cycle.

The third cycle, started with the best culture of the second cycle, is also given in detail in table 6 in order to strengthen these points and show that, with increasing enrichment, the positive cultures are more clearly differentiated from those containing background mutants.

Altogether, nine cycles were necessary to reach, from the original 10^{-7} concentration, a concentration of 2% resistants. At this stage, the best culture of the last enrichment cycle was plated on ordinary agar, and 317 colonies subcultured from it; they were then individually tested for resistance at the standard chloramphenicol concentration. Four colonies were found to be resistant, with a clearcut differentiation from the sensitives (see fig. 1).

Indirect selection having thus been fully achieved, it remains to be seen whether the rate of enrichment corresponds to that expected if all the resistants counted are spontaneous mutants. The incomplete survival of the resistant at the standard drug concentration interferes with this calculation. The actual survival of the indirectly selected mutant, however, could be estimated directly as soon as the mutant was isolated in pure culture: it proved to be variable from day to day, but constantly dependent on the frequency of sensitives which, if present at a high concentration, proved to provide some protection to the resistants. A typical experiment with mixtures of sensitives and resistants is given as follows:

Counts on mixtures of resistants and sensitives

2 plates at each level

No. of plated						
Resistants		200	200	200	200	200
Sensitives		10^8	10^7	10^6	10^5	—
Counts on non drug-agar		—	—	—	—	228; 170
Chloramphenicol 18 μg/ml agar		34; 70	6; 4	3; 0	0; 9	0; 1

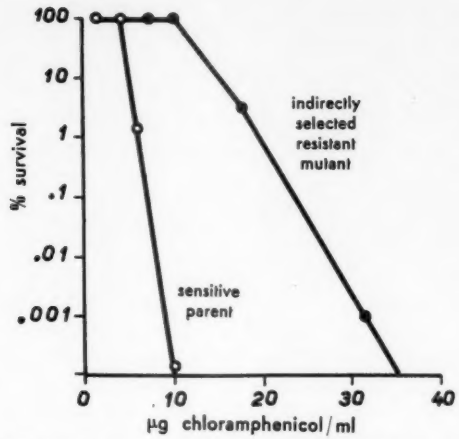

FIGURE 1.—Survival curves on chloramphenicol agar plates of sensitive parent culture and an indirectly selected resistant mutant.

TABLE 7

Indirect selection for chloramphenicol resistance in E. coli

1	2					3	4	5		
Cycle	No. of positive cultures					Max. no. of resistants	Ratio dilution/ plate assay	χ^2	df	*P*
	2	1	½	¼	⅛					
I	?	1	*1*	0	0	216	1.1 ± 0.8	.8	3	non signif.
II	3	*1*	1	0	2	814	3.0 ± 1.0	10.6	4	5–2%
III	4	3	3	*2*	0	940	3.8 ± 1.1	3.8	4	non signif.
IV	10	9	*8*	4	0	3350	21 ± 4.6	4.0	4	non signif.
V	9	6	3	3	*1*	10,700	9.9 ± 2.2	0.9	4	non signif.
VI	3	*1*	1	1	0	52,750	1.7 ± 0.7	1.4	4	non signif.
VII	9	9	*6*	1	3	1.68×10^5	15.4 ± 3.4	4.9	4	non signif.
VIII	7	1	1	0	*1*	2.19×10^6	3.3 ± 1.0	5.5	4	non signif.
IX	*6*	4	1	0	0	4.2×10^6	3.8 ± 1.1	2.4	4	non signif.
								34.3	35	50%

Column 1: Enrichment cycle. Column 2: No. of "fertile" cultures in each of the five series of 10 tubes, each series being seeded with the number of resistant bacteria indicated at the top of each subcolumn (estimated by previous plate assay). Column 3: No. of resistant bacteria in 0.1 ml of the best "fertile" culture, i.e. the culture which was found to contain the highest number of resistant mutants by plate assay on chloramphenicol agar and was used for the next enrichment cycle. Such a culture belongs to the series indicated by italics in column 2. Column 4: Ratio between dilution assay of resistant mutants and plate assay. Column 5: Analysis of the validity of the dilution assay.

A summary of the results obtained in the nine enrichment cycles is given in table 7. The value given in the 4th column, i.e. the ratio between the resistants found by the test in liquid medium and the resistants, given by the assay on chloramphenicol agar plates may deserve some comment. It is obtained by considering that the proportions of fertile tubes at the various relative concentrations of 2, 1, ½, ¼, ⅛ constitute, in fact a "dilution assay" of the numbers of resistants in the culture to be enriched.

Estimation was carried out by maximum likelihood, and the fifth column (table 7) contains an analysis of the validity of the assay by the calculation of an (approximate) value of χ^2 for each cycle. Total χ^2 is 34.3 with 35 d.f., showing a good fit.

The ratios given in the 4th column of table 7 agree with the previously noted fact that plate counts tend to underestimate the numbers of resistants. The values estimated by dilution assay are more reliable, but unfortunately are subject to high standard errors, as is well known. This makes computation of enrichment at every step hardly worth while. However, some information can be obtained from the total enrichment observed. In nine enrichment cycles, the concentration of resistant organisms went up from 10^{-7} to 0.023, i.e. there was a total enrichment, whose logarithm is 5.37 (this figure may, if any, be higher than the real one, being based on the best samples at every step).

The expected enrichment at every step can be calculated, as before, assuming equal growth rates for normals and mutants, from n/m, where n is the number of tubes into which the sample is distributed and m the number of bacteria seeded, which is given at the head of the appropriate subcolumn of column 2.

Such a value is, however, the estimate by the previous plate assay, and this, we have seen, is deficient by a factor given in column 4. The number m can therefore be obtained by multiplying the estimate from plate assay (top of the corresponding subcolumn, column 2) by the corresponding value in the fourth column. The total expected enrichment is obtained by summing the logarithms of the expected enrichments at each cycle and equals 6.1 ± 1.1.

The magnitude of this standard error does not permit a detailed comparison of the total observed and expected enrichment. The latter should be greater than the former if $k < 1$; it may therefore be predicted that probably the growth rate of the mutant is slightly smaller than that of the sensitive. In view of counting difficulties of the resistant types in mixtures, no direct examination of the relative growth rates of mutant and parent in mixtures was carried out.

A comparison of the relative resistance to chloramphenicol of the normal parent and of the indirectly selected mutant by plate assays is given in figure 1. After 25 daily transfers in broth, with a multiplication factor of 10^4 at every transfer, for a total of 332 generations, there is no variation in resistance.

DISCUSSION

One of the first cogent lines of evidence on the adaptation controversy was LURIA and DELBRÜCK's (1943) finding of a high variance from culture to culture in the assay of resistant mutants. The conclusion that this variance was genetic, and arose from the stochastic event of mutation, was countered by the supposition that the

environment varies uncontrollably from one culture tube to the next. The bacteria would then differ in physiological state, and hence in their responses when later confronted with the drug. Experiments in this paper show how this question can be elaborated, by a test of the "heritability" of the variance, when samples from "high" and "low" cultures are regrown in fresh medium. These experiments were not specifically designed for the purpose, but as far as they go, they agree precisely with the expectation that "high" cultures already contain many stably resistant cells and "low" cultures do not. To give the obtained result that inocula from "high" cultures generate further "high" cultures, the resistance would have to be heritable in a new set of culture tubes, in conflict with the supposition of purely physiological variability. Whether physiological accidents in the culture tubes of plain broth ultimately cause individual mutational events is not in question here; the issue is whether such variations arise and persist independently of the drug.

The replica-plating and indirect selection procedure has provided still more direct evidence of preadaptation, as drug-resistant mutants can be isolated that have not experienced the drug (LEDERBERG and LEDERBERG 1952; BRYSON and SZYBALSKI 1955). However, certain precautions are required for the correct interpretation of negative results. For example, DEAN and HINSHELWOOD (1953) report having prepared plates of synthetic medium carrying 2×10^7 single colonies (sic) of *Bact. lactis aerogenes*. The plates were then replicated in two passages to chloramphenicol agar, and "the congruent site on the corresponding non-drug plate (about five colonies) was picked off". Needless to say, the further experience with this isolate gave no evidence of the presence of a mutant clone: the limit of resolution of replica plating is perhaps 0.1% of the area of a plate, not $5/2 \times 10^7$ after two passages. These authors, and LAM and SEVAG (1955) have also overlooked the occurrence of mutations during the growth of colonies which had been shown to contain resistant cells by replica plating. As with other populations of bacteria, the colony may contain resistant cells in only very small proportions, as GOLDSTEIN, MAGASANIK and UMBARGER (1955) have explicitly shown with the same material. The method of indirect selection in liquid medium described in the present paper is somewhat less liable to these troubles, as it is possible to distinguish small and large clones, which are not readily distinguished in replica platings.

Unlike replica plating, limit sampling is subject to precise quantitative formulation, though all the subtleties of population dynamics must be taken into account. The success of the procedure testifies that all of the resistant cells that are counted in the assays are already in the population being tested. The method will, of course, break down in situations, as can be devised, where the selective action of the drug is limited or delayed so that further growth and new mutations may ensue on the test plates. When either method of indirect selection should appear to fail, it should at least be shown that it is applicable to the recovery of already adapted clones artificially added to the tested populations, if the negative result of indirect selection has to testify against preadaptation.

The successful application of indirect selection to chloramphenicol resistance lends no support to the possible reservation that while single step, high level resistance is preadaptive, multistep resistance of a lower order may be directly induced by a

drug (as discussed by EAGLE 1955). However, we must exclude from our discussion phenomena of survival or persistence which entail no genetically stable variations in the survivors. For example, DEAN (1955) reports that "cultures prepared from colonies on proflavine plates are no more resistant than a normal organism". It is plausible that physiological adaptations are concerned here, and they may also be involved in the biochemical defenses which genetically resistant organisms can raise against antibiotic attack. These are, unfortunately, very obscure and we know very little about whether these defenses are, in a physiological sense, adaptive or constitutive. For the purpose of this discussion, our operational definition of resistance is the ability of a cell to survive exposure to the drug which, like the other inherent potentialities of the organism, is defined by the genotype.

SUMMARY

Indirect selection of resistant mutants, i.e. in the absence of the toxic agent or drug can be carried out not only by replica plating but also by a procedure making use of subcultures in liquid media, and choosing the best subcultures by testing samples of them on drug media. A repetition of this process can lead to pure resistant lines which have never been in contact with the drug or toxic agent, thus proving the spontaneous origin of resistants by mutation. The procedure can be speeded up considerably by the subdivision of limiting dilutions containing one or few resistant organisms. Although more time-consuming than replica plating, this procedure is more easily amenable to quantitative analysis, to conclude whether resistant mutants obtained by direct selection (in presence of the drug) are all, or in part, the result of spontaneous mutation. Our conclusions from experiments carried out on first step streptomycin and chloramphenicol resistance in *Escherichia coli* are in agreement with the hypothesis that all resistant mutants on drug plates have a spontaneous origin. The observed rate of enrichment of resistant mutants in indirect selection has been comparable with that which was expected on the basis of the experimental conditions, and of growth rate differentials between mutant and parent.

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A CYTOGENETIC STUDY IN MAIZE OF DEFICIENCY-DUPLICATION PRODUCED BY CROSSING INTERCHANGES INVOLVING THE SAME CHROMOSOMES[1]

D. M. GOPINATH AND C. R BURNHAM

Tobacco Research Institute, Vedasandur via Dindigul (S. Rly), India and Department of Agronomy and Plant Genetics, Institute of Agriculture, University of Minnesota, St. Paul 1, Minnesota

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MULLER (1930) was unsuccessful in his attempt to produce viable individuals with a deficiency or a duplication for certain chromosome segments from crosses between translocations involving the same chromosomes in Drosophila, but suggested it as a valuable method to "determine the effects of genic disproportions of all portions of the germplasm and to compare these with the effects of gene mutations in the same region." It was one of the methods used by PATTERSON *et al.* (1937) to produce hyper- and hypoploid individuals for their study of X chromosome balance. BLAKESLEE *et al.* (1936) also used it as one method of producing phenotypically changed plants in *Datura stramonium*. They pointed out that the phenotypic effects of the addition of blocks of genes without change in chromosome number were similar to differences frequently observed between species.

GOLDSCHMIDT (1950) expressed the opinion that the real genetic units of the chromosomes are sections of different sizes as evidenced by the study of salivary gland chromosomes in Drosophila. Translocations, inversions, or viable deficiencies in the "yellow" section of the X chromosome produce the same phenotypic mutation. CATCHESIDE (1947) has presented evidence for the presence of a duplication or a deficiency in occasional progeny of a translocation heterozygote in *Oenothera blandina*, and accounts for their origin by unequal crossing over. He has listed the various possible types of duplications, and discussed their behavior as to crossing over in the duplication heterozygotes. Viable deficiencies and duplications have been shown by other workers also to simulate certain known mutations, e.g. STADLER (1933) and MCCLINTOCK (1938, 1941, 1944) in maize, and BRIDGES (1917) in Drosophila. Duplications may be a source of new gene material, within which subsequent mutations may occur.

The ability to produce individuals carrying duplications for certain chromosome segments may be useful to the plant breeder; for example, would the duplication of a potent gene for disease or insect resistance increase the resistance? A duplication for an endosperm type such as sugary or waxy might be expected to produce changes in the chemistry of these types. Two members of a true multiple allelic series might be combined in a homozygote in this manner; whereas ordinarily they could both be present only in the heterozygote. The subsequent behavior of such experimentally produced duplications might furnish information on "pseudoallelism". One method

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of producing duplications experimentally is by crossing interchanges as reported here. Another is by crossing inversions which overlap (BEADLE and STURTEVANT 1935).

The purpose of this study was to consider the theoretical results to be expected from crosses between interchanges involving the same chromosomes, to study the cytological behavior in such intercrosses in maize and to determine if deficiencies and duplications can be located and established in this manner.

The expected theoretical results depend on the relative positions of the break points in the two interchanges with reference to each other and to the centromeres, and are shown in table 1. They are divided into two main groups: # 1 in which the breaks in either or both chromosomes are in opposite arms, and # 2 in which both breaks in each of the chromosomes are in the same arm. It will be noted from the last three columns that the F_1 gametes with the new non-parental combinations of interchanged chromosomes carry both deficiencies and duplications in all types of intercrosses except 2b and 2c. In certain cases the deficient spores may be viable. In types 1a and 1c the deficiencies and duplications are for the translocated end segments. In type 1b they are for the translocated segments of one chromosome and the "between breaks" segment of the other chromosome. In types 2b and 2c one of the

TABLE 1

The possible types of intercrosses between two translocations, Tc and Td, involving the same two chromosomes. The break loci, the constitutions of the F_1, and the segments which may be duplicated or deficient in the possible non-parental chromosome combinations are shown. The parallel vertical lines between 3, 4 and 9, 10 represent the centromeres

Type	Break positions in translocations c and d		F_1 interchanged chromosomes	Non-parental combinations are		
	First chromosome	Second chromosome		No.	Deficient for	Duplicated for
1a	1 2 3 | 4 5 / c d	6 7 8 9 | 10 11 / c d	Tc C = 6 7 2 3 4 5 C^1 = 1 8 9 10 11 Td D = 1 2 3 4 11 D^1 = 6 7 8 9 10 5	(1*) (2*)	1, 11 5, 6, 7	5, 6, 7 1, 11
1b	1 2 3 | 4 5 / c d	6 7 8 9 | 10 11 / d c	Tc 6 7 2 3 4 5 1 8 9 10 11 Td 1 2 3 4 6 5 7 8 9 10 11	(1) (2)	1 5, 7	5, 7 1
1c	1 2 3 | 4 5 / c d	6 7 8 9 | 10 11 / c d	Tc 6 2 3 4 5 1 7 8 9 10 11 Td 1 2 3 4 6 5 7 8 9 10 11	(1) (2)	1 5	5 1
2a	1 2 3 | 4 5 / c d	6 7 8 9 | 10 11 / c d	Tc 6 2 3 4 5 1 7 8 9 10 11 Td 6 7 3 4 5 1 2 8 9 10 11	(1) (2)	7 2	2 7
2b†	1 2 3 | 4 5 / c d	6 7 8 9 | 10 11 / d c	Tc 6 7 2 3 4 5 1 8 9 10 11 Td 6 3 4 5 1 2 7 8 9 10 11	(1) (2)	— 2, 7	2, 7 —
2c	1 2 3 | 4 5 / c d	6 7 8 9 | 10 11 / c d	Tc 6 2 3 4 5 1 7 8 9 10 11 Td 6 7 2 3 4 5 1 8 9 10 11	(1) (2)	7 —	— 7

* From the non-parental combination. No. 1 is C + D^1; No. 2 is D + C^1.
† See figure 1.

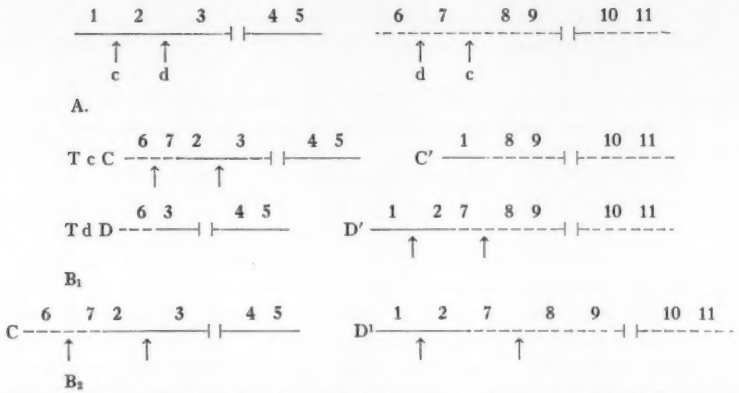

FIGURE 1.—Diagrams illustrating the type 2b intercross in which the c translocation involves a shorter piece of one chromosome, but a longer piece of the other than does the d translocation. A = break positions in original chromosomes. Tc = c translocation. Td = d translocation. B_1 = F_1 from cross of Tc × Td; CC^1 are the new chromosomes from Tc; DD^1 from Td. B_2 = combination of non-parental chromosomes ($C + D^1$) which has a duplication for both the regions between the two break points. The $D + C^1$ combination which is deficient is not shown.

non-parental combinations has a duplication but no deficiency, and should result in viable spores. Intercrosses of this type appear to be the most valuable therefore for the experimental production of duplications. The type 2b cross is shown in figure 1. Here the $C + D^1$ combination of chromosomes has a duplication for the "between breaks" segment in each of the two original chromosomes. In this type the first translocation involves a shorter piece of one chromosome, but a longer piece of the other than does the second translocation. In type 2c the breaks for both translocations in one chromosome are at the same point, the breaks in the other one may be as they are in 2a or in 2b. In a species with directed segregation, if the F_1's of these intercrosses showed "pairs" of chromosomes at meiosis, there would be no mechanism for directed segregation; and sterility should be about 50% if both non-parental chromosome combinations aborted. The degree of spore abortion to be expected in F_1 would depend on whether any of these deficiencies were viable. In a species not having directed segregation, e.g. maize, this would decrease the sterility over that observed in the parental heterozygotes. Observations on the degree of spore abortion (pollen and ovule) in the F_1 of such crosses constitutes an easily applied test to determine if deficiencies may be viable. In types 2b and 2c, reduced spore abortion should be the rule, since one of the cross combinations has a duplication and no deficiency.

The cytological configurations expected in F_1 depend on the extent to which the transposed homologous segments pair in their new positions. The possible ways in which pairing might occur may be derived by inspection of table 1, column #4. In type 1a, complete homologous pairing might be accomplished in complicated three-dimensional figures. "Pairs" in F_1 might be of two types, depending on whether

the homologous "between breaks" segments or the homologous ends paired. Studies of pairing in intercrosses of this type between a series of translocations involving the same two chromosomes with breaks at different points should furnish useful information on the forces governing pairing, e.g. the points at which pairing is initiated.

In F_1 types 1b and 1c, if the homologous #6 end segments pair the internal 2-3-4 segments of these chromosomes are in reverse order with respect to each other (see table 1). Inversion pairing followed by crossing over could produce further duplications plus deficiencies. This is similar in principle to the mechanism suggested by BRINK and COOPER (1932) for reduced crossing over in certain translocations.

In F_1 of type 2c at pachytene, if "pairs" are formed, each should show a loop equal to the length of the one "between breaks" segment. The term "loop" is used to refer to the cytological configuration resulting when one "homologue" is longer than the other and the ends and the remainders of the chromosomes are paired. It may result from one member of a pair carrying either a deficiency or a duplication. In F_1 of types 2a, 2b, and 2c, the two "pairs" might have an intercalary association between them if the homologous portions of the two loops pair. These configurations have been observed by BOROJEVIC (1954).

At pachytene in type 2a if pairs are formed in the F_1, the ends are homologous but the intercalary "between breaks" segments are not. A loop configuration should be formed whose length should be the difference in length between the two "between breaks" segments. At times both segments might show lack of pairing. Both chromosome "pairs" should show the loop.

In F_1 of type 2b at pachytene, if "pairs" are formed, each should show a loop equal in length to the total length of the two "between breaks" segments.

Associations of four chromosomes had been expected in F_1 in which the recognition of deficiencies or duplications near the break points might be difficult due to variable degrees of pairing. To avoid this, the F_1's were backcrossed to both homozygous parents. The resulting progeny should include plants similar to the F_1, others homozygous for the recurrent parental translocation, and others with a non-parental combination of the translocated chromosomes. The latter should be homozygous for one or the other of the two translocated chromosomes from the recurrent parent, the other "pair" having the transmissible duplication or deficiency and showing the loop configuration at pachytene. If transmission of the duplication through the pollen occurs, plants homozygous for the duplication should be obtainable by selfing. These would be tetrasomic for that segment. That these are the expected results may be determined by referring to figure 1.

EXPERIMENTAL RESULTS

The first intercrosses were made in 1946 by the second writer. From the list of homozygous translocations then available and the information on break positions, pairs of translocations were selected which seemed to fit the specifications for each of the groups with the added requirement for group 2 that the breaks for the two translocations be close together in at least one of the chromosomes, and thus improve the chances that a deficiency for the short segment between them would be viable.

TABLE 2

Genetic and cytological data on breakage positions in the translocaions used

Translocation	Chromosome	Locus of break	Linkage	Chromosome	Locus of break	Linkage	Source of information*	
							Gen.	Cytol.
T1-7a	1	L.42	*br*-.5-*T*-2.5-*f*	7	L.17	*T*-1.1-*ra-gl*	1	1
T1-7b	1	L.54	close to *br*	7	S.16	*T*-0.9-*ra-gl*$_1$		7
T1-9a	1	S.17	*P*-21.2-*T*-35.6-*br*	9	L.17	*c-wx*-11.2-*T*	4, 2	7
T1-9c	1	S.61	*ts*$_2$-*P*-0.8-*T*	9	L.32	*c-wx*-12.7-*T*	4, 2	7
T2-6b	2	S.69	*gl*$_2$-3.9-*T*-0.9-*B*	6	L.49	*Pl-sm*-3.3-*T*	8, 6	7
T2-6(6049)	2	S.7	†close to *lg gl*$_2$	6	L.23	†close linkage with *Y*	9	9
T3-7a	3	S.23	*ts*$_4$-5.0*d*-20.2-*T*-15.9-*lg*$_2$	7	L.17	†*ra*-1.1	3	7
T3-7b	3	S.9	*T-d*-0.4-*lg*$_2$	7	L.03	near *ra*	3	7
T4-5c	4	S.45	*su*-1.1-*T*	5	L.38	*bm*-3.5-*T*-15.5-*pr*	5	7
T4-5d	4	S.21	*su*-1.9-*T*	5	L.19	*bm*-2.5-*T*-5.5-*pr*	5	7
T5-7a	5	L.75	*bm-pr*-15-*T*	7	L.71	*ra-gl*-22-*T*	9	9
T5-7c	5	L.38	*bm*-8-*T*-4-*pr*	7	L.71	*ra-gl*-40-*T*	9	7

* (1) BURNHAM 1948. (2) ANDERSON 1938. (3) ANDERSON and BRINK 1940. (4) ANDERSON 1941. (5) ANDERSON, KRAMER, and LONGLEY 1955 (chrom. 4). (6) ANDERSON, KRAMER, and LONGLEY 1955 (chrom. 6). (7) LONGLEY 1950. (8) PATTERSON 1952. (9) BURNHAM unpublished.

† Order in the linkage group not known or uncertain

The intercrosses were made, but only those thought to belong to group 2 were used in this study. Each parental translocation was crossed also with a standard normal stock to furnish a check on the sterility when they were heterozygous and to furnish material to use as a check on the deficiency tests. Seven of the intercrosses did not show reduced spore abortion: *T2-6c* × *d*, *T2-6(6052)* × *c*, *T3-9b* × *c*, *T4-9a* × *b*, *T5-6a* × *b*, *T5-6a* × *c*, and *T5-6b* × *c*. Six intercrosses had reduced spore abortion and were selected for further study. The translocations involved in these latter intercrosses, together with the breakage positions in the linkage maps and in the chromosomes are shown in table 2. Based on this information, the crosses: *T1-7a* × *T1-7b*, *T1-9a* × *T1-9c*, and *T4-5c* × *T4-5d* were expected to belong to group 2a; *T3-7a* × *T3-7b* to group 2b; and *T2-6b* × *T2-6(6049)*, and *T5-7a* × *T5-7c* to group 2c. The breaks may not be at the exact points listed, since the cytological determinations at pachytene are subject to some error. The supplemental genetic linkage data in the same table were used in planning the genetic tests.

A summary of the data on pollen abortion in the homozygous and heterozygous translocation parents, and the ovule and pollen abortion in the F_1 plants of the crosses selected for further study are in table 3. In all the intercrosses in that table, pollen or ovule abortion was less than the average of that observed in plants heterozygous for the parental translocations.

The pollen of F_1 plants from *T1-7a* × *T1-7b* was practically normal, but ovule abortion was higher than normal, 11.3% in F_1 vs. 5.2 for the parents. Ovule and pollen abortion in the F_1's from *T4-5c* × *T4-5d* did not differ from normal. This is expected if the two parents had identical translocations. Cytological evidence to be presented later indicates *T1-7a* and *T1-7b* are different, but that for *T4-5c* and *T4-5d* is not conclusive. It appears that, in all these intercrosses, certain of the expected spore combinations failed to abort. For the genetic tests for deficiencies, F_1 plants from the intercrosses were used as the female parents in crosses with the available

TABLE 3

Pollen abortion percentages in homozygous and heterozygous translocations and pollen and ovule abortion in F_1 plants from crosses between translocations involving the same two chromosomes

Translocation	Pollen of				% abortion in F_1 cross		
	Homozygote		Heterozygote		Pollen		Ovules average
	% aborted	No. of plants	% aborted	No. of plants	Average	Range	
T1-7a	5.1	5	47.8	4	3.6	2.0–7.4	11.3
T1-7b	5.2	3	50.0	4			
T1-9a	1.4	6	50.0	6	45.0	30.0–50.0*	31.1
T1-9c	7.0	8	43.5	6			
T2-6b	4.8	4	48.6	11	27.2	20.0–35.0	37.2
T2-6(6049)	6.7	7	35.0	8			
T3-7a	8.2	3	36.6	3	23.8†	20.0–30.0	29.7
T3-7b	6.0	6	50.0*	—			
T4-5c	4.0	5	43.7	4	3.9	0–8	2.0
T4-5d	4.3	2	50.0*	—			
T5-7a	0		47.3	5	12.0	11.0–4.0	24.4
T5-7c	2.8		48.0	7			

* Estimated.

† Small but partially filled, large and small completely empty were included as aborted.

recessive characters located in the chromosome arms involved in the translocations. Reciprocal crosses were made in most cases to provide material for checking transmission through the pollen. The plants with low pollen abortion among the progeny from the F_1's backcrossed to either parent were likewise crossed with the recessive testers in many cases. Plants with low pollen abortion were selected in the field based on estimations with a 40× pocket microscope, tassel samples being preserved for later checking and counting. Plants heterozygous for the parental translocations were crossed to a limited extent with the recessives as a check on the results. The cross of *T5-7a* × *T5-7c* has been studied in detail by BOROJEVIC (1954). Further data on it are not reported here.

The results obtained from the genetic tests for deficiencies in F_1 plants from the intercrosses, and in plants from backcrosses of the F_1 to the parental translocations are summarized in tables 4 and 5. Only three intercrosses gave recessives in F_1. Two of these are shown in table 4. The one omitted is *T1-9a* × *T1-9c* which in the first test with a *br f* stock gave *br* plants and *f* plants. Subsequent tests showed that part of the plants of at least one of the parents had been heterozygous for these markers. Two F_1 plants of the cross *T1-7a* × *T1-7b* when crossed as the female parent with fine stripe (*f*) gave fine striped plants in F_1, 29 in a total of 249 plants. The reciprocal cross gave no *f* plants. These results suggest the transmission of a defi-

TABLE 4

Results from genetic tests which indicate the functioning of a gamete carrying a deficiency for f *in chromosome 1 in* T1-7a × T1-7b *and for* fl_2 *in chromosome 7 in* T3-7a × T3-7b. *Check results are included*

Crosses	No. of F_1 plants tested	No. of plants in progeny	
		Normal	Recessive
Checks:			
(*T1-7a* × N) × *br f bm*	3	253	0
(N × *T1-7b*) × "	4[1, 2]	127	41 *br*
Intercrosses.			
(*T1-7a* × *T1-7b*) × *f*	2	220	29 *f*
f × (*T1-7a* × *T1-7b*)	1	196	0
(*T1-7a* × *T1-7l*) × *br f*	1[1]	129	5 *f*, 53 *br*
" × *br f bm*	7[1, 2]	349	23 *f*, 165 *br*
(*T3-7b* × *T3-7a*) × fl_2	1	110	15 fl_2

[1] Part of the F_1 plants tested were heterozyogus for *br*.

[2] Data taken by E. TURCOTTE from later tests of the same stocks.

TABLE 5

Negative results obtained from genetic tests for deficiency the number of plants in each test being indicated after each gene. All plants tested for transmissible deficiency were used as the female parents except those with the number underlined

Translocation stocks tested	First chromosome	Second chromosome
T1-7a × *T1-7b*	*sr*-76; *as*-75; zb_4-12; *an*352; *ad*-10; bm_2-235	*ra*-295; *gl*-<u>151</u>; v_3-295; *bd*-73
T1-9a × *T1-9c*	*sr*/+1157; zb_4-127	
T1-9c × *T1-9a*/*T1-9c**	zb_4-35; *an*-94; bm_2-112	
T2-6(6049) × *T2-6b*	gl_2-232; v_4-232	*pb*-65; su_2-157; *py*-34; *py*/+-130
T3-7a × *T3-7b*	*d*-170; lg_2-159; ra_2-338	*ra*-576; *gl*-564; *ij*-77†; $v_3$488; *bd*58
T4-5d × *T4-5c*	*wl*-82; gl_3-<u>274</u>; bm_3-<u>226</u>	sh_3-620; gl_3-216; *bm*-193; *bv*-451
T4-5c × *T4-5d*/*T4-5c**	*wl*-311; bm_3-83	

* From these backcrosses, plants with low pollen abortion were selected for testing. They were probably similar to the F_1 of the intercross.

† In one test with only twelve plants, there was one *ij* type plant; none in the other larger test

ciency for that locus through the female parent and its non-transmission through the pollen. Another plant of the intercross was tested as the female parent with *br f*. This segregated for *br* and for *f* but no *br f* plants. The low frequency of *f* is similar to that in the first two crosses. Subsequent tests of the homozygous and heterozygous *T1-7a* parent stock showed them to be homozygous for the normal alleles of these characters. Tests of the *T1-7b* stock showed that some of the plants were heterozygous for *br* and bm_2 but none carried *f*. This accounts for the anomalous results in the F_1 for the characters other than *f*. In subsequent tests of seven more F_1's of the intercross crossed as female parent with *br f bm*, all but one segregated *br* or bm_2 or both. All but one also gave low frequencies of *f*; a total of 21 in 542 plants. There were no *br f* plants, indicating that the functional deficiency did not include the *br* locus.

The second case in table 4 was for floury-2 endosperm, an unlinked character. When a plant with low pollen abortion from the backcross progeny of *T3-7b*/+ × (*T3-7a* × *T3-7b*) was crossed as the female parent with floury-2, 15 kernels in a total of 125 were floury. Since the *T3-7a* × *T3-7b* F_1 had been used as the male parent in the backcross, it is probable that the backcross plant used to give this result was also similar to the F_1, i.e. heterozygous for the two translocations. Unfortunately the F_1 of this cross had not been tested with fl_2. The results indicate that floury-2 should be in chromosome 3 or 7. The necessary check crosses between these same recessives and plants heterozygous for the parental translocations were not available, but since this character had been used very little in crosses, it probably was not present.

The results from all the other genetic tests for deficiency were negative. These included tests of the parental translocation heterozygotes with many of the same genes that were tested on the intercross heterozygotes. Only the female transmission tests of the intercrosses are included in table 5, except for a few in which the test through the male was the only one made (underlined). These latter are not conclusive since the deficiency may not have been male transmissible. For some of the tests, the numbers are too small. The fact that the pollen abortion in these F_1 intercrosses was lower than the average for the two parental translocations is evidence for the presence of deficient but viable combinations in each of these crosses. It is probable that the genetic tests did not detect them because the genes used were not at the proper loci.

CYTOLOGICAL ANALYSIS

The F_1 plants from crosses between translocations involving the same two chromosomes, as listed in table 2, and the progeny from the backcrosses were examined at the diakinesis and pachytene stages of meiosis. The results are summarized in table 6.

At diakinesis, ten "pairs" was the only configuration observed in the F_1 plants of all the different intercross combinations.

Plants from the F_1 crosses and from backcrosses of the F_1 to both parental translocations were available for most of the combinations and were examined at pachytene. Unfortunately, for most of the backcrosses, the F_1 had been used as the male parent, thus decreasing the chances that any of the progeny might have come from a functional deficiency plus duplication gamete from group 2a or a duplication plus duplication gamete from group 2b. The expectation in the backcross is therefore a 1:1 ratio of plants homozygous for the recurrent parental translocation and plants similar to the F_1. Although more difficult to detect, in no case was there any indication of a configuration of four chromosomes at pachytene. The standard pachytene lengths used are based on unpublished data from McCLINTOCK and our own pachytene studies. They differ slightly from those given by LONGLEY (1939) and republished by RHOADES (1950). The numbers of sporocytes per plant with at least one of the two chromosomes showing a loop, given in the last column of table 6, is to a certain extent an index of relative frequency except that the extremely high numbers for the *T2-6* intercrosses were due to a larger number of cells having been examined.

The results for the two intercrosses which gave positive genetic tests for deficiency will be discussed first, *T1-7a* × *T1-7b* and *T3-7a* × *T3-7b*. In each of the five F_1

TABLE 6

Summary of cytological observations on the F_1 of translocation intercrosses or on the F_1 backcrossed to the parent translocations

Cross	No. of plants examined	Diakinesis	Pachytene loop present in		Observed number of cells per plant with loop
			First chromosome	Second chromosome	
T1-7a × *T1-7b*	5	10 II	none	all plants	2, 3, 6, 6, 9
T1-7b × F_1	10	10 II	none	6 plants	1, 2, 4, 5, 7, 7
T1-7a × F_1	10	10 II	one plant*	3 plants	1, 1, 4
T1-9a × *T1-9c*	5	10 II	3 plants	2 plants	3, 11, 12
T1-9c × F_1	8	10 II	3 cells of one plant	2 plants	2, 13
T1-9a/+ × F_1	8	10 II†	none	none	
T2-6(6049) × *T2-6b*	7	10 II	none	2 plants	1, 2
T2-6b × F_1	7	10 II‡	4 plants	6 plants	11, 16, 16, 22, 29 (or forks)
2-6(6049)/+ × F_1	8	10 II†	3 plants	4 plants	1, 8, 11, 70
T3-7a × *T3-7b*	3	10 II	T-config.	T-config.	7, 6 with T-config.
T4-5c × *T4-5d*	10	10 II + 'B'	none	none	
T4-5c × F_1	4	10 II + 'B'	2 plants§		

* One cell with loop, one other doubtful.

† Those with high pollen abortion and a ⊙4 not listed.

‡ In 4 of the plants the chromosome 6 "bivalent" consisted of a long and a short homologue; this chromosome pair had a loop or an unequal-appearing bivalent.

§ A loop in one cell of each plant, the chromosomes not identifiable.

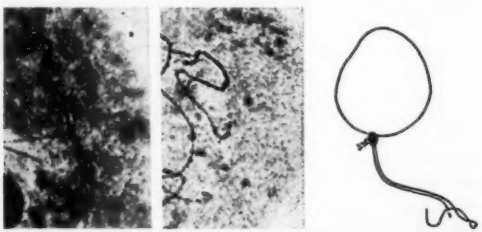

FIGURE 2a.—(left) Photomicrograph showing loop on chromosome 7 from *T1-7a* × *T1-7b*. b.—(center) Photomicrograph showing loop on chromosome 9 from *T1-9a* × *T1-9c*. c.—(right) Camera lucida drawing showing unequal lengths of chromosome 6 from *T2-6(6049)* × *T2-6b*.

plants from *T1-7a* × *T1-7b* which were examined, a few sporocytes showed a loop on the long arm of chromosome 7 near the centromere. Figure 2a is a photomicrograph of a typical loop observed in chromosome 7. The configurations indicate that both breaks are in the long arm of 7, the one in *1-7b* not in the short arm as listed. If the breaks were in opposite arms as listed, the configurations should not be those observed. Similar observations were made on at least 7 of 20 plants examined in progenies from backcrosses to the parental translocations in which the F_1 was used as the male parent. The plants not showing any loop in these chromosomes were probably the translocation homozygotes. No loop was observed in chromosome 1 in

any of these plants. A loop in chromosome 1 is expected in the F_1 plants showing it in chromosome 7, (table 1, types 2a, b, or c). In a plant from a backcross to *1-7a*, one cell showed a loop in the long arm of 1 considerably distal to either supposed point of breakage. The loop is not expected to be a long one, only about .1 of the long arm of the chromosome. Its absence in 7 in most of the cells and in 1 in all but one of the cells examined may be due to frequent compensatory pairing, i.e. a stretching of one chromosome and a contraction of its homologue resulting in the absence of the loop. This might occur more frequently in chromosome 1 since the arm in which the loop was expected is considerably longer than the long arm of 7. Another factor may be proximity to the centromere, the one in 7 being closer to the centromere than is the one expected in 1. The recessive *f* plants in F_1 were not examined cytologically. This is planned in current experiments.

Cytology of T3-7a × T3-7b

Only three plants were available for cytological study for the F_1 cross *T3-7a* × *T3-7b*. "Pairs" were observed at diakinesis in the F_1 plants. They showed no intercalary loop in either chromosome 3 or 7 at pachytene. The pachytene configurations for chromosome 7 were frequently forked or T-shaped. No such configuration was seen in chromosome 3, although the diakinesis photographs suggest that a longer "pair" had a shorter member.

Based on the breakage points as listed in table 2, one arm of one member of each "pair" should have been much longer than the other, about 50% longer for chromosome 3 and about seven times as long for 7. The T-shaped or forked configurations indicate that pairing did not begin at the ends or even simultaneously at the ends and the centromeres in these chromosomes of unequal length. Also based on the breakage points as listed this cross should belong to group 2b from which a duplication plus duplication or a deficiency plus deficiency non-parental combination gamete would be expected. The fact that the genetic test for deficiency gave about 13% of floury-2 seeds should indicate that a deficiency-carrying gamete had functioned, a deficiency for a relatively long intercalary segment of the short arm of 3 and a shorter segment of 7.

Cytology of T1-9a × T1-9c

In the F_1 plants from *T1-9a* × *T1-9c*, bivalents were observed at diakinesis in all sporocytes. At pachytene, a loop was seen in the short arm of chromosome 1 in three plants and in the long arm of 9 in two plants (fig. 2b). In 9, the longer chromatid had a fold-back in that position in several cases.

Of the eight plants from *T1-9a*/+ × F_1 and eight from *T1-9c* × F_1, only two from the former showed any evidence of loops (the plants with a ⊙4 were discarded). In one plant two different chromosomes showed a loop and were identified as interchanged chromosomes 1 and 9. At diakinesis in one plant the chromosome 1 "pair" was unequal in length.

Cytology of T2-6(6049) × T2-6b

At diakinesis in the seven F_1 plants of *T2-6(6049)* × *T2-6b*, ten "bivalents" were observed in all cells examined. At pachytene, only two of these plants showed any

evidence of loops. These were large loops in the long arm of chromosome 6. More extensive observations were made on the backcross plants.

In the seven plants from the backcross of *T2-6b* × F_1 only one showed no loops in either 2 or 6. In four of the other plants there was a loop or forked configuration on 2. In these and two others the pachytene configurations of chromosome 6 were variable. According to the break points as listed, the short arm of one of the members of the chromosome 2 "pair" should have been about 25% longer than the other, and the long arm of one of the members of the 6 "pair", about 50% longer. In most cases, one of the homologues of chromosome 6 was much longer than the other, the extra piece remaining unpaired or folded back on itself to give T-shaped and other configurations (fig. 2, c). In others the distal ends paired and there was an intercalary loop, the position of which varied widely. These indicate that pairing in this chromosome was initiated at the end in part of the cells. Similar behavior was observed in plants from the backcross on *T2-6(6049)*.

Results from T4-5d × T4-5c

The pachytene configurations in most slides of these F_1 plants were well spread permitting a critical study of the chromosomes. No loops were observed. The normal fertility of the F_1 suggests that the two translocations may be identical. According to the break points as listed in table 2, the two members of each "pair" in F_1 should be of equal length, but with considerable lack of homology. In the backcross of the F_1 on *T4-5c* only two sporocytes showed any evidence of a loop, but the chromosomes could not be identified. The genetic information from linkage tests shows about the same values with *su*, and the breaks in 5 are both between *bm* and *pr*. However, in the *T4-5c* heterozygote, the crossover value between *bm* and *pr* was about 19% while in *T4-5d* it was only 8%. The two are probably not identical but the breaks may be closer together than indicated in table 2.

DISCUSSION

One of the intercrosses, *T1-7a* × *T1-7b*, produced F_1 plants with normal pollen and only slightly lower than normal ovule fertility (11% abortion), and yet at pachytene there was in many sporocytes a loop in chromosome 7. No loop was observed in chromosome 1, but the genetic tests showed that the F_1 produced some viable chromosome combinations deficient for the *f* locus in that chromosome. Only the pachytene cytology and this genetic result indicate that these two translocations are not identical. They are known to have different origins, *T1-7a* having occurred naturally (BURNHAM 1930) and the *T1-7b* by X-rays several years later (ANDERSON 1935). For the intercross of *T4-5c* × *T4-5d*, evidence from spore fertility, diakinesis and pachytene cytology (with two possible exceptions), and the genetic deficiency test did not indicate that they are different. Negative genetic evidence is not critical as a test. Transmission tests of the new chromosome combinations, using genetic markers should furnish further information.

In Datura, BLAKESLEE *et al.* (1937) reported that all the hybrids between the first prime type 2 and fourteen other P.T.2 races from various parts of the world "formed equal bivalents" and were considered identical. The results presented here indicate that other tests are needed to establish identity.

In cases where "pairs" are observed in F_1 of an intercross, unexpected behavior in the progeny might occur as the result of illegitimate crossing over in regions paired non-homologously, resulting in additional duplication or deficiency. In a plant carrying a duplication, unequal crossing over within the duplication may give rise to new types as pointed out by CATCHESIDE (1947).

The genetic tests reported appear to indicate that in maize a viable intercalary deficiency may be produced by crossing translocations which involve the same two chromosomes and have breaks in the proper relative positions. Viable intercalary duplications should be producible by the same method. Since types 2b and 2c (table 1) are the most useful crosses for their production, the importance of accurately locating interchange break points both cytologically and in the linkage maps is obvious. With that information, the proper interchanges can be selected to obtain the desired duplications.

SUMMARY

The theoretical results to be expected from crossing two interchanges which involve the same two chromosomes are presented. The two new combinations of chromosomes in the intercross may carry a deficiency plus duplication or a duplication plus duplication for "between break" or translocated segments, depending on the relative positions of the breaks with respect to each other. In one type, one combination should always carry a duplication and no deficiency. In maize and in other species without directed segregation, if one of the new spore combinations is viable, the F_1 of the intercross will have lower spore abortion than the average of the two heterozygous interchange parents. Six of the thirteen intercrosses tested in maize had this lower degree of spore abortion.

The F_1 of *T1-7a* × *T1-7b* had normal pollen fertility and only slightly lower than normal ovule fertility (11% abortion). This F_1 when crossed as the female parent with a fine striped stock (*f*) produced 11.2% of *f* plants, indicating a low frequency of spores that were probably deficient and yet able to function. The reciprocal test produced no *f* plants, indicating (to the extent tested) that the deficiency was not functional through the pollen. In all the F_1 plants of this cross part of the sporocytes showed a loop in the long arm of chromosome 7 near the centromere, indicating a deficiency or duplication. Although expected, no corresponding loop was found in chromosome 1. Without the pachytene cytology and the genetic test showing a deficiency these two interchanges might have been classed as having breaks at identical loci. One other F_1, *T3-7a* × *T3-7b*, also gave low sterility in F_1 and about 13% of floury-2 seeds (chromosome 7) in the deficiency tests. No intercalary loops were observed at pachytene but chromosome 7 was frequently asynapsed at the distal end of the long arm.

None of the other four intercrosses with lower sterility gave any recessive offspring in the deficiency tests for the genes used. It is probable that the genes tested were not at the deficient loci. Loops were observed in chromosomes 1 and 9 in part of the cells in the cross of *T1-9a* × *T1-9c*. In the *T2-6(6049)* × *T2-6b* intercross backcrossed to both parents a loop or a forked configuration was observed in both chromosomes. One member of chromosome 6 was longer than the other. In the *T3-7a* × *T3-7b* intercross a T-configuration (never a loop) was observed in both chromosomes.

In certain chromosomes pairing may be initiated at the ends, in others not. The *T4-5c* × *T4-5d* cross was normal in fertility, no loops were observed in chromosomes identified as 4 or 5, and the deficiency tests gave only normal offspring. The two may not be identical in spite of these results.

A method by which duplications for known regions can be produced could be of use to the plant breeder and experimentalist. By inference from the results reported here, the translocations furnish such a method in maize. To make full use of it, accurate information on the break points is necessary.

NOTE ADDED TO PROOF

In Drosophila two papers, one of which appeared since this paper was submitted and referred to the other, have reported viable individuals with new combinations of Chromosome segments. One method used in their production was crossing individuals with translocations involving the same chromosomes.

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ENZYMATIC STUDIES OF THE PHYSIOLOGICAL GENETICS OF GUINEA PIG COAT COLORATION. I. OXYGEN CONSUMPTION STUDIES[1, 2]

MORRIS FOSTER

Whitman Laboratory of Experimental Zoology, The University of Chicago and Osborn Zoological Laboratory, Yale University

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THE GENETICS of coat color pigmentation in the guinea pig have been extensively analyzed by WRIGHT (1916, 1917, 1925, 1927). These studies have been supplemented by various attempts to discover the underlying inherited physiological processes, either by quantitative determinations of pigmented end product (E. S. RUSSELL 1939; HEIDENTHAL 1940; WRIGHT and BRADDOCK 1949; WRIGHT 1949) or by assays for enzyme (dopa oxidase) activity in the skins of a wide variety of genotypes (W. L. RUSSELL 1939; GINSBURG 1944). A detailed consideration of these different types of studies is contained in WRIGHT's review (1942). The study reported here represents an additional attempt to ascertain inherited melanin-forming enzyme variations in guinea pig skin, and some of these results have already been summarized (FOSTER 1952b).

W. L. RUSSELL's histochemical studies with incubated frozen skin sections involved visual grading of degree of blackening both in hair bulb and in basal epidermal melanocytes. His assays for dopa oxidase activity indicated a strong correlation between the intensity of the hair bulb reaction (but not that of the basal epidermal melanocytes) and the amount of natural yellow pigment (phaeomelanin) controlled by the genotype. A similar correlation was reported by GINSBURG, who performed turbidimetric assays for dopa oxidase activity in fresh and stored ("delayed") skin extracts (1944). Our own results, while in many respects confirming those of RUSSELL and GINSBURG, tend on the whole, but with important exceptions, to indicate a correlation between genetically controlled amount of dark pigment (eumelanin) and tyrosinase or dopa oxidase activity. However, as will be discussed later, at least some of the major apparent discrepancies between reported results can be reconciled on the basis of differing materials and methods.

MATERIALS AND METHODS

Materials

The foetuses used in this study came from the stocks of PROFESSOR SEWALL WRIGHT, and the genotypes were determined by us on the basis of the foetal phenotypes and of the genotypic possibilities of each specific mating as determined by

[1] These studies were performed mainly at the University of Chicago during the tenure of a postdoctorate research fellowship from the National Cancer Institute, National Institutes of Health, with additional help from the Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. The subsequent work at Yale University was supported in part both by an institutional research grant from the American Cancer Society, administered by the Yale University Committee on Atypical Growth, and by NSF Grant G-517 from the National Science Foundation.

[2] The technical assistance of MRS. GREVILDA S. TREMBLY and subsequently that of MR. EDWARD D. BANKS is gratefully acknowledged.

PROFESSOR WRIGHT'S examination of breeding records and the phenotypes of each pair bred. The major coat color allelic series are as follows:

1. *E* (predisposition for dark pigmentation), *e* (predisposition for yellow pigmentation), e^p (dark and yellow spotted).
2. *A* (agouti or yellow banded), *a* (solid dark color hair).
3. *B* (black pigment), *b* (brown pigment).
4. Albino series alleles; *C*, c^k, c^r, c^d, c^a. (Combinations lacking gene *C* show different degrees of reduction of dark and yellow pigments).
5. *F*, *f* (*ff* reduces amount of yellow pigment only, in presence of *P*; appreciable reduction of dark pigment only in association with *pp*).
6. *P*, *p* (pink-eyed dilution factor; *pp* always dilutes dark pigment, never yellow). Nearly complete absence of dark pigment in the double recessive, *ffpp* (pale cream phenotype). On a yellow (*ee*) background, however, *ffP-* is equivalent to *ffpp*.
7. *S*, *s* (*ss* gives white spotting).

The various genotypes studied by us were as follows:

Black (*E-* or e^p- *aaBCF* or *ffP*)
Black agouti (*EABCFP*)
Brown (*EaabbcF* or *ffP*)
Pale brown (*EaabbCFfpp*)
Pale sepia (*EaaBCFpp*)
"Red", most intense of yellow series (*ee*——*CFP*)
Pale cream (*E—bbC–ffpp*)
Lower albino series alleles, i.e.;
  Black (*EaaBc^{k}c^{r}FFP*)
  Dark sepia (*EaaBc^{r}c^{r}FFP*)
  Light sepia (*EaaBc^{r}c^{a}FFP*)
  Pale sepia (e^pe^p*aaBc^{k}c^{k}pp*)
  Albino (—c^ac^a—) or nearly white (*eec^{d}c^a*)

Methods

A Warburg constant volume respirometer was used to measure oxygen consumption as a measure of the enzymatic oxidation of tyrosine or dopa. The enzyme preparations were simply dorsal skins ground to a fine powder while frozen, as previously described (FOSTER 1951, 1952a). Because of the difficulty in demonstrating enzymatic activity in skins after birth, the dorsal skins of approximately 50 day old foetuses were used. At this foetal age (the date of the dam's previous litter taken as the date of conception) pigmentation is intense, the hairs have already emerged from the skin and the skin itself is ground in the frozen condition without much difficulty. As an additional check against gross errors in estimating foetal age the crown to rump length of each foetus was determined. This length varied between 7.5 and 10 cm for foetuses ranging in estimated age from 50 to 54 days. As an additional check on the classification of the foetal genotypes in segregating litters, a small sample of skin from each foetus was fixed in 1:10 formol-alcohol (½ hour), dehydrated in absolute alcohol (½ hour), cleared in benzene (at least one hour) and mounted in Permount (Fisher Scientific Co.). Skin homogenates not used on the day of preparation were stored at −20°C, and such homogenates stored as long as 8 months retained their characteristic

activities. In preparation for a Warburg run, homogenized dorsal skin from a single foetus was suspended in a measured amount of pyrex redistilled water and aliquots were pipetted into Warburg reaction vessels. The amount of skin per ml aliquot ranged in various experiments from $\frac{1}{20}$ to $\frac{1}{10}$ of a dorsal foetal skin.

The pyrex reaction vessels had a capacity of approximately 10 ml. The center wells contained 0.2 ml of 20% KOH, the main part of each vessel contained 1 ml of skin suspension, while the side arm contained 0.5 ml of M/10 phosphate buffer, pH 6.8, or substrate dissolved in an equal volume of buffer. The substrate solution consisted of L-tyrosine (0.5 mg/ml). Occasionally L-dopa (0.5 or 1.0 mg/ml) was used as exogenous substrate to check the results with tyrosine. When the enhancing agent, iodoacetamide, was used, it was dissolved in the buffer (with or without substrate) at a concentration of 6 mg/ml.

After temperature equilibration at 38°C (10–15 minutes), side arm contents were tipped into the flasks (*zero time*).

Endogenous oxygen uptake controls differed from the corresponding experimental vessels only in the absence of substrate. The *net oxygen uptake curves* were obtained by subtracting the mean endogenous oxygen consumption of the controls from the mean oxygen consumption of the experimental vessels for each reading. All flasks, experimentals, controls and thermobarometers, were run in duplicate. When substrate autoxidation controls were run, 1 ml of 0.9% NaCl was substituted for skin homogenate in the main portion of the reaction vessel.

Control homogenates in the absence of iodoacetamide showed a marked tendency to increase their oxygen consumption rates abruptly after several hours of incubation, sometimes even surpassing the oxygen consumption in experimental vessels. For this reason it was decided to rely primarily on net oxygen uptake computations obtained from experiments run in the presence of iodoacetamide, where oxygen consumption irregularities were very infrequently encountered.

Measures of tyrosinase and dopa oxidase activities were obtained by estimating maximum rates of net oxygen uptake. In the case of tyrosine, where an induction period was evident, the maximum rate was taken from the straight line portion of the S-shaped curve. The induction period was determined by extending the straight line portion until it intersected the time axis. The pooled results of our experiments reveal a significant negative correlation ($r = -0.65$) between maximum rate and induction period. In the case of dopa, with no induction period, maximum net oxygen uptake rates were obtained from the first two sets of readings, generally within the first ½ hour of each experiment. In the case of certain very weakly active skins, such as "Red" or some agouti skins, oxygen consumption measurements could not be obtained, although a slow darkening over controls was noted on overnight incubation. A rate of 1 microliter/hr was arbitrarily assigned to these skins to indicate the existence of a low level of activity.

RESULTS

Demonstration of tyrosinase activity

Manometric and histochemical demonstrations of tyrosinase activity in hair bulbs of skin fragments have already been reported (FOSTER 1952a). Other criteria for

demonstrating the enzyme included specificity of the skin catalyst for the L-configuration of tyrosine. Furthermore, the catalyst was poisoned by the addition of phenylthiourea. Finally, both pigment production and oxygen consumption (especially the shortening of induction periods) were enhanced by iodoacetamide, presumably by destruction of inhibiting sulfhydryl compounds. A typical set of net oxygen uptake curves is shown in figure 1. In this case the skin of a 52 day old pale brown foetus had been used.

Comparison of activities of various genotypes

The results of individual experiments, involving the maximum net oxygen uptake of self-colored and agouti skins with tyrosine in the presence of iodoacetamide are shown in the form of frequency distributions in figure 2. These results, together with those obtained with dopa, as well as the results with tyrosine-negative skins, are summarized in table 1. Analysis of the results in table 1 reveals the following:

1. Substitution of *ff* for *F*

Although *ff* intense brown and black skins appear to show somewhat higher activity than corresponding *F* skins, the differences are not significant. These results

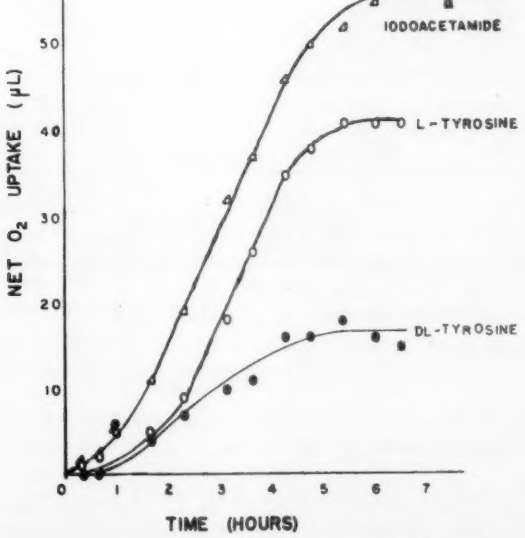

FIGURE 1.—Net oxygen consumption curves for a pale brown skin (*bbFfpp*) from a 52-day foetus, showing specificity for the L-configuration of tyrosine and enhancement of activity by iodoacetamide, especially in shortened induction period.

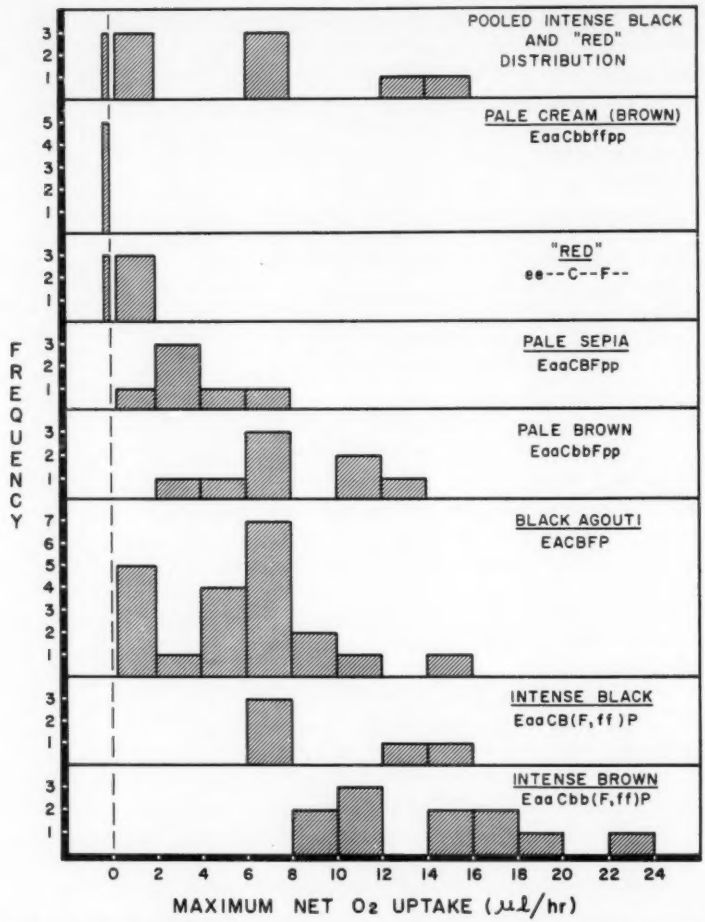

FIGURE 2.—Frequency distributions of tyrosinase activity measurements (maximum rate of net oxygen uptake in microliters/hr) of tyrosine- and/or dopa-positive skins of different genotypes.

were therefore pooled in order to permit other comparisons, and the pooled data are enclosed in the space bounded by double lines.

2. Substitution of *bb* for *B*

The average activity of the pooled intense browns towards tyrosine is greater than that of the pooled intense blacks, but not significantly so (the value for t gives a P value near the 10% level). The average activity of the pale browns toward tyrosine, however, is significantly higher than that of pale sepias. From these two sets of

TABLE 1

Tabulated summary of tyrosinase activity in various genotypes, as measured by maximum rate of net oxygen consumption in the presence of iodoacetamide

Genotype	Phenotype	Tyrosine			Dopa (.5 mg/ml)			Dopa (1 mg/ml)		
		M	SE	(N)	M	SE	(N)	M	SE	(N)
EaaCbbffP	Intense brown	15.9	± 2.5	(5)	39.7	± 1.6	(5)	68.5	± 4.6	(5)
——*B*——	Intense black	13.6		(1)	40.5		(1)	58.5		(1)
——*bbF*—	Intense brown	12.8	± 1.2	(6)	32.7	± 10.2	(2)	45.2	± 12.7	(2)
——*B*——	Intense black	9.2	± 2.2	(4)		—			—	
EaaCbb (F,ff)P	Intense brown	14.2	± 1.3	(11)	37.7	± 2.8	(7)	61.8	± 6.0	(7)
——*B*———	Intense black	10.1	± 1.9	(5)	40.5		(1)	58.5		(1)
——*bbFpp*	Pale brown	8.3	± 1.2	(8)	23.4	± 2.6	(4)	31.0	± 6.4	(3)
——*B*——	Pale sepia	3.7	± 0.9	(6)	18.6	± 1.4	(4)	27.2	± 2.6	(4)
ee-C—F—	"Red"	0.5		(6)	7.1	± 1.0	(3)	9.5	± 0.9	(4)
EaaCbbffpp	Pale cream brown	0.0		(5)	5.9	± 1.8	(5)	9.6	± 2.4	(6)
EACBFP	Black agouti	5.7	± 0.8	(21)		—			—	
c^kc^r, c^rc^r, c^kc^kpp, c^rc^a, c^ac^o, c^rc^o.	Black to white	0		(7)	0		(2)	0		(1)

comparisons it seems reasonable to conclude that a mutation from the black to the brown pigmented condition results in somewhat more demonstrable tyrosinase activity. This change runs counter to the change in amount of pigment, since brown hair contains only about 40% of the amount found in black hairs (WRIGHT 1949).

3. Substitution of *pp* for *P*

The average activity of intense browns towards tyrosine is significantly higher than that of pale browns, and the same holds true for a comparison of intense vs. pink-eyed blacks (pale sepias). Thus mutation from intense dark coloration to the pale, pink-eyed, condition involves parallel reductions in both enzyme activity and amount of eumelanin.

4. Substitution of lower albino series alleles for *C*

In our experience with the manometric technique the absence of gene *C* results in the complete loss of activity towards exogenous tyrosine or dopa, regardless of the amount of natural dark pigment present in the skin. For example, c^kc^r intense blacks are nearly phenotypically indistinguishable from the corresponding tyrosine-positive C- blacks, yet, paradoxically, their behavior in Warburg reaction vessels appears to be categorically different.

Two apparently exceptional cases were noted in relation to the lower albino series group. One foetus of a litter involving possible segregation of *ppC* pale sepias and

ppc^r*c*^{r} very pale sepias was classified as a very pale sepia. The skin of this foetus, however, in contrast to the tyrosine-negative results with all other skins of this group, gave a weakly positive reaction. PROFESSOR WRIGHT has kindly advised me that this foetus could well have been an abnormally light pale sepia, *ppC*- with additional incompletely defined dilution factors. Thus this case does not appear to contradict our other findings in the lower albino series group. We have not, however, included this case among the usual pale sepias, inasmuch as it would be an exceptionally weak variant in that category.

The other apparently exceptional case appears to have involved a misclassification. A dark foetus, originally classified as intense black (i.e., *C-B-*) gave no reaction towards tyrosine, in contrast to the marked activity of every other intense *C-B-* black. A histological comparison of a whole mount skin sample from this foetus with samples obtained from *C* and *c*^{k}*c*^{r} intense blacks indicated that the exceptional foetal skin was lighter than these intense blacks. PROFESSOR WRIGHT, on further examination of his breeding records, has informed me that segregation for sepia of genotype *c*^{r}*c*^{a} could have occurred in this litter. Thus a reasonable explanation is available for each of the apparently exceptional cases encountered.

5. Substitution of *ee* for *E*

"Red," or the most intense yellow, skins gave nearly negative results with tyrosine. Only a slow overnight darkening, and only in the presence of iodoacetamide, was evident in 3 out of 6 skins. A correspondingly low activity towards dopa was also observed. Thus the inherited color change from intense black or brown to "red" is accompanied by a drastic reduction in tyrosinase activity.

In order to determine whether the low demonstrable activity of red skin might be due to the presence of large amounts of sulfhydryl inhibitors, the effects of increasing the concentration of iodoacetamide were tested. No aditional enhancement was obtained, thus suggesting only low level of enzyme activity in red skin.

6. Substitution of *A* for *aa*

Of the 21 cases in the agouti group 15 were classified as agoutis on the basis of a histological examination of their skin samples, which showed a mixed population of black and red hair bulbs. The remaining 6 foetuses, obtained from a black self (*aa*) × agouti (*Aa*)mating, had been tested before the procedure of preparing whole mount skin samples had been adopted. The mean activity and the range for these 6 skins however, were nearly the same as for the other 15 identified skins. (Mean for the 6 unidentified skins: 5.5 microliter/hr, 5.7 microliter/hr for the 15 identified ones. Range for the 6 unidentified skins was 1.0–14.0 microliter/hr, 1.0–10.8 microliter/hr for the 15 identified ones.) It thus appears justifiable to pool these results.

The average activity of agouti skins towards tyrosine was significantly lower than that of self-colored *C* blacks. Examination of the frequency distribution of agouti skin activity (fig. 2) indicates two additional striking features—an extremely wide range and a bimodal distribution. Such a range and bimodality are approached by pooling the results obtained with the self-colored *C* blacks and "reds," as shown at the top of figure 2. Thus it appears that the net reaction of an agouti skin tends to fall into

one of the two major categories—an activity level characteristic of self-colored black, or a level characteristic of solid "red."

7. Factor interaction in *ffpp* (pale cream) genotypes

Of the 5 pale cream skins tested none showed activity towards tyrosine, and only the very weak activity towards dopa (significantly higher than the autoxidation level of about 2 microliter/hr) suggested the presence of a small amount of enzyme. As far as our data go, it appears that pale cream skins are even less active than "red" skins.

Especially striking is the phenomenon of factor interaction between genes *ff* and *pp*, affecting the level of demonstrable enzyme activity. Both *ff* intense brown and *pp* pale brown skins exhibit, respectively, very high and high levels of activity. In the double recessive pale cream (*ffppbb*) combination, however, enzyme activity has practically vanished. That is to say, enzyme activity in either of the two mutant types is high, but is practically eliminated when the two mutations in homozygous condition are combined in the same organism.

DISCUSSION

The dark-yellow problem

Our results, in relation to the dark-yellow problem fall into three categories:

1. Parallelism between tyrosinase activity and dark pigmentation

The parallel effects of gene substitution on tyrosinase activity and on the amount of natural dark pigment are evident in the cases of the *P* and *F* loci. Substitution of *pp* for *P* (in the presence of *F*) reduces both enzyme activity and amount of dark pigment, although it does not affect the amount of yellow pigment. Moreover, substitution of *ff* for *F* (in the presence of *P*), while reducing yellow pigment, reduces neither tyrosinase activity nor the amount of dark pigment.

The complementary relation between *ff* and *pp* in the nearly dopa-negative double recessive pale cream skin, with factor interaction affecting both eumelanin (but not phaeomelanin) and enzyme activity, suggests in addition the existence of some as yet unspecified metabolic connection between the synthesis of tyrosinase and the synthesis of yellow pigment. Clarification of this relationship must await more definite information concerning the enzymatic production of yellow pigment.

2. Reasonably explained cases

a. Although substitution of *bb* for *B* results in a marked decrease in amount of dark pigment, this genetic alteration certainly does not decrease and even seems to increase the activity towards exogenous tyrosine. If brown pigment is produced from tyrosine, reduced pigmentation can be explained by postulating some form of endogenous substrate limitation, with no effect on total tyrosinase activity, as was previously suggested by WRIGHT (1942). With a smaller portion of total enzyme combined with endogenous substrate in brown skin than in black, the greater activity of brown skin could then be due to the greater amount of enzyme available to act on exogenous substrate.

b. If dark and yellow pigments are produced from different precursors (granting

the existence of unspecified metabolic links between these two major pigmentary systems), the marked difference in tyrosinase activity between black and yellow skins needs no explanation. On the other hand, on the hypothesis that there is a parallel effect of gene substitution on the capacity to produce yellow pigment and on tyrosinase activity, the change from *E* to *ee* or from *aa* to *A* should involve no change in tyrosinase activity, since these gene changes involve no intensity modification of yellow pigmentary capacity. Yet our observations indicate striking changes in activity resulting from these gene substitutions. It is still possible, however, to assume with WRIGHT (1942) that tyrosine is a common precursor of both eumelanin and phaeomelanin and that significant divergence of the eu- and phaeo- melanic processes occurs at a stage in the reaction sequence subsequent to the oxidation of tyrosine. Experimental support of this view is furnished by the report that C-14 labeled tyrosine or some product of this is incorporated into phaeomelanic granules in the mouse (MARKERT 1955), although it has not yet been determined whether the labeled tyrosine is incorporated into the pigmented or non-pigmented portion of the yellow granules.

3. Unexplained

Clearly, the paradoxically tyrosine- and dopa-negative results obtained with the lower albino series group do not permit any rationalization of the lack of parallelism between tyrosinase activity and amount of dark pigment. In the absence of positive results with the manometric method two different possibilities may be entertained: 1) either tyrosinase is present in these skins, in which case the necessary conditions for its demonstration are drastically different from the conditions necessary for its demonstration in *C*-skins, or 2) in the absence of gene *C*, an enzyme system other than the tyrosinase system might produce dark pigment from tyrosine or some other chromogen.

Thus, although there are difficulties in considering the *C* locus, the observed effects of gene substitution at the other five major color loci together with the case of factor interaction between *ff* and *pp* either directly support, or can be reconciled with, the view that the tyrosinase system produces dark pigment, that yellow pigment might be produced from some chromogen other than tyrosine (although this question is very much open), and that these two major pigmentary systems are somehow metabolically linked.

Comparison with results of previous investigators

Although our conclusions are quite different from those advanced by W. L. RUSSELL and by GINSBURG, the various sets of observations show a good deal of agreement. Moreover, at least some of the differences in results can be attributed to differences in materials and methods.

Comparison with RUSSELL'S results

The visual grading of the dopa reaction, while having the advantage of permitting observation of a localized reaction, suffers from three major shortcomings as contrasted with the manometric method: (1) It involves shifting standards for comparing activities of differently colored skins, i.e., black vs. yellow, pale sepia or pale

cream. (2) A linear scale of visual grades probably corresponds to exponential increases in amounts of pigment (WRIGHT and BRADDOCK 1949). (3) Dopa is much more readily transformed into melanin than is its more stable precursor, tyrosine. (On the other hand, the visual grading method is probably more sensitive in the detection of weak reactions on lightly pigmented backgrounds.) Thus this assay method probably imposes a lower ceiling on the observer than does the manometric method, and this lower ceiling value is more often reached when dopa rather than tyrosine is used as the exogenous melanogenic substrate.

As regards visual assays for hair bulb reactions, it is not surprising, then, that while there is a general correspondence with our results, there is a general relative upgrading of the less active skins. Thus yellow hair bulbs seem only slightly less active than black or brown, with no apparent reduction in pale (*pp*) genotypes, and various lower albino series allelic combinations exhibit only reduced activity as compared with the absence of demonstrable activity with the manometric method. It is therefore possible to reconcile all our major results except in the case of *ff* intense genotypes, where RUSSELL reported a sharp reduction in enzyme activity while no such reduction was observed manometrically.

As for the dopa reaction of the basal epidermal melanocytes the dark-yellow problem vanishes, since these cells appear to contain only eumelanin, even when the coat color is yellow (W. L. RUSSELL 1939). The correspondence between these and our results is almost perfect, such as greatly reduced or no reaction in the cases of lower albino series combinations, *pp* and *ffpp* genotypes, as well as the presence of full activity in *ff* dark genotypes. In our preparations of homogenized foetal skin, however, the vast majority of the melanocytes are to be found in the hair bulbs. (DR. WILLYS K. SILVERS has kindly shown me histological preparations of dorsal skin from a 55-day foetus, and the numerical preponderance of hair bulb melanocytes is obvious.) Thus the interesting possibility arises that the hair bulb melanocytes of foetuses might behave enzymatically more like those of the basal epidermal class than of the hair bulb class in animals at least 30 days after birth.

Comparison with GINSBURG's results

Our results, with two apparent exceptions, agree well with those reported by GINSBURG for his "immediate" extracts, i.e., no reaction in the absence of gene *C*, sharply reduced activity on substitution of *ee* for *E* and the lack of reduced activity on substitution from *B* to *bb*. The only important apparent differences are found in the cases of *ff*, where GINSBURG noted reduced activity, and of *pp*, where he reported no reduced activity—the reverse of our results. However, reductions due to single or double doses of gene *f* were most readily observed by GINSBURG in yellow (*ee*) genotypes, which we have not yet studied. Moreover, the apparent lack of reduced dopa oxidase activity in *pp* extracts might be due to a lower ceiling of measurable effect in the turbidimetric method.

Additional storage of GINSBURG's skin extracts in a refrigerator ("delayed" extracts) resulted, for all cases excepting unpigmented skin, in increased production of dopa melanin, thereby bringing them into greater correspondence with RUSSELL's results involving hair bulb reactions. Thus some of the lower albino series skin ex-

tracts, inactive as "immediate" extracts, became active as "delayed" extracts. (Similar results have recently been reported by HOROWITZ and FLING, 1954, in connection with extracts from Drosophila.) The similarities and differences between our results and these are about the same as obtain in the comparison between our results and RUSSELL's hair bulb assays. Here again some of the differences may in part be due to the use of dopa as exogenous substrate in one study and of tyrosine in the other.

The agouti pattern

The yellow-banded agouti color pattern has been the subject of various recent investigations, and it seems desirable to summarize and correlate the results of these studies. Substitution of gene *A* for *aa* results in a pigmentary pattern characterized by a subterminal phaeomelanic band in an otherwise dark hair. There is thus a strict correlation between the stage of the hair growth cycle and melanocyte function. The histological studies of E. S. RUSSELL (1949) in the mouse lead to the view of a reversible switch mechanism involved in agouti banding, a replacement of the eumelanic process by the phaeomelanic process (rather than a temporary unmasking of yellow pigment) followed by a resumption of the dark pigmentary process. The transplantation studies of SILVERS and RUSSELL (1955) indicate that the kind of pigment produced by hair bulb melanocytes is determined by the follicular environment, which in turn is conditioned by genes at the agouti locus in the mouse.

As regards the biochemical aspects, DANNEEL (1949), reviewing his and other studies on rabbits, viewed the yellow band as a possible consequence of a temporary inhibition of dopa oxidase activity or of a temporary drastic reduction in the rate of dopa oxidase synthesis. FOSTER (1951), comparing the oxygen consumption patterns of solid yellow, solid brown and brown agouti mouse skin homogenates incubated in tyrosine, suggested that yellow band formation might be due to the rather abrupt suspension and resumption of tyrosinase activity as a consequence of sharp fluctuations in the level of a tyrosinase inhibitor. Our present guinea pig data, however, do not lend themselves as readily to such a conclusion, because the corresponding guinea pig genotypes differ in their oxygen consumption patterns from those of the mouse in that, in all cases so far tested, guinea pig skin homogenates incubated in tyrosine exhibit an induction period. Suggestive evidence for the implication of sulfhydryl inhibitors has been reported by CLEFFMANN (1953) in the agouti rabbit. Moreover, CLEFFMANN (1954), growing mouse skin in tissue culture, reported both the induced production of yellow pigment in black self skin by the addition of glutathione to the medium and the partial suppression of yellow pigment formation in agouti skin by the addition of sulfhydryl-destroying iodoacetamide to the medium.

Correlations between yellow band formation and stage of hair growth cycle are provided by DANNEEL (1949) and CLEFFMANN (1953), who reported that in the rabbit the yellow band occurs in a thickened region of the hair. Moreover, CLEFFMANN (1953) noted that this period of increased growth rate in agouti rabbit hair is accompanied by an increased mitotic index in the cells of the hair matrix, although no comparable control data are given for corresponding developmental stages of solid black hairs. Finally, a characteristic feature of the keratinization process in hair

growth is the oxidation of —SH groups to form cross linking disulfide groups (see GIROUD and LEBLOND 1951).

An attempted synthesis of the above observations leads to the tentative view that the gene controlled period of yellow band formation is marked by some rhythmic deviation in the keratinization process, with greater production of —SH compounds in the melanocyte environment than occurs in the case of non-agouti hair. The temporary increase in —SH groups inhibits tyrosinase activity and, perhaps, tyrosinase synthesis as well, thereby temporarily diverting pigment formation in the direction of phaeomelanin production. The introduction of —SH inhibitors into the melanocytes might be part of a reciprocal exchange between melanocytes and the developing hair cells migrating past them, the melanocytes "inoculating" the hair cells with pigment (see CHASE, RAUCH and SMITH 1951) and the —SH compounds passing from hair cells into melanocytes, perhaps via the same dendritic processes. The difficulty in visualizing the gene-controlled mechanisms involved in the onset and termination of this rhythmic pattern producing fluctuation still remains, however.

CONCLUSIONS

The major obstacle to the formulation of a scheme of gene substitution effects is our ignorance concerning the nature of the phaeomelanic process. A second obstacle is the apparently paradoxical behavior of lower albino series combinations. Whether it be assumed that the eumelanic and phaeomelanic processes diverge from a point of common substrate or of common enzyme precursor, it still appears necessary to attribute additional properties to component parts of these processes (such as thresholds, competition, inhibition, differences in reaction rates, formation of active or inactive complexes, or alternate pathways) in order to harmonize assumed changes in single-step reactions with observed phenotypic effects. Thus on the assumption of common enzyme precursor one complication arises in considering the effects of *ff* and *pp*, acting separately on the synthesis of a yellow producing enzyme or of tyrosinase, respectively, and also interacting to affect the eumelanic system. On the assumption of common melanogenic substrate, the dual effects of albino allelic substitutions on both eumelanic and phaeomelanic processes introduce a complication. In any event, in this study involving attempts to insert a metabolic phenotype between genotype and phenotype observed at higher organizational levels, it appears that the various gene substitutions affect various components of a ramifying metabolic network. Thus some mutations appear to affect primarily the rate of tyrosinase synthesis (i.e., *e* and *p*), another mutation might somehow affect the amount of available tyrosine (*b*), another the periodic excess of a tyrosinase inhibitor (*A*) in the melanocyte environment, still another might differentially affect both tyrosinase synthesis and phaeomelanin production (*C* series), yet another might affect primarily the phaeomelanic process (*f*), and finally two different biochemical lesions (*ffpp*) somehow additionally affect tyrosinase synthesis.

SUMMARY

1. Inherited variations in the tyrosinase system in foetal guinea pig skin, in which hair bulb melanocytes greatly outnumber basal epidermal melanocytes, have been

studied by means of oxygen consumption measurements of individual skin homogenates incubated in tyrosine and also, less frequently, in dopa.

2. The observed effects of gene substitution are as follows: (a) Replacement of *P* by *pp*; which reduces the amount of dark pigment (eumelanin), but not of yellow pigment (phaeomelanin); results in decreased tyrosinase activity. (b) Replacement of *F* by *ff*; which reduces the amount of phaeomelanin, but not of eumelanin; results in no decreased tyrosinase activity. (c) Simultaneous replacement of *F-P-* by *ffpp*; involving factor interaction causing virtually complete elimination of eumelanin, but not of phaeomelanin; results in no demonstrable tyrosinase activity and barely demonstrable dopa oxidase activity. (d) Replacement of *B* by *bb*; which reduces the amount of eumelanin, but not of phaeomelanin; results in no decrease in tyrosinase activity. (e) Replacement of *E* by *ee*, involving the substitution of yellow for a dark coat, results in a drastic reduction in tyrosinase activity to a barely demonstrable level. (f) Replacement of *aa* by *A*, involving subterminal yellow bands, results in a very wide range and a bimodal distribution of tyrosinase activity measurements. The tendency of these measurements of black agouti skins to cluster about the values characteristic of solid yellow and of solid black skins suggests that the net activity measured for each agouti skin is a function of the relative number of hair bulb melanocytes engaged in yellow pigment formation at the time of enzyme assay. (g) Replacement of *C* by any combination of lower alleles of the series, with differential diluting effects on both eumelanin and phaeomelanin, results in the apparently complete absence of both tyrosinase and dopa oxidase activities. These paradoxically negative results, especially in such intensely black skins as $c^k c^r$, might in part be due to a high threshold for enzyme demonstration inherent in the manometric technique. Drastically reduced activity in all skins lacking gene *C* is, however, certain.

3. When allowances are made for differences in materials and methods, there is quite good agreement between the present results and those previously obtained by W. L. RUSSELL (1939) and by GINSBURG (1944).

4. The present results, however (except for the *C* series), can be considered as either directly supporting or at least reconcilable with the view that the manometric method measures inherited capacity to produce dark pigment. Directly supporting evidence is found in the cases of *ff*, *pp* and *ffpp* genotypes. The lack of reduced activity in brown skin, however, seems to require the assumption of endogenous substrate limitation resulting from substitution of *bb* for *B*. The very slight tyrosinase activity of yellow hair bulbs (in *ee* or *A* skins) may be interpreted in two ways: (a) yellow pigment is derived from a chromogen other than tyrosine or (b) yellow pigment is derived from tyrosine, but that significant divergence of the eu- and phaeomelanic processes occurs at some stage subsequent to tyrosine oxidation. Finally, the observed factor interaction on the chemical level in pale cream skin (*ffpp*) indicates the existence of some metabolic link between the synthesis of tyrosinase and the production of yellow pigment, thus supporting previously advanced conclusions based on genetic evidence (WRIGHT 1916).

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IDENTIFICATION OF HALF-TRANSLOCATIONS PRODUCED BY X-RAYS IN DETACHING ATTACHED-X CHROMOSOMES OF DROSOPHILA MELANOGASTER[1]

SEYMOUR ABRAHAMSON, IRWIN H. HERSKOWITZ, AND H. J. MULLER

Department of Zoology,[2] Indiana University

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ONCE THE detachment by X-rays of attached-X chromosomes in oocytes of *Drosophila melanogaster* carrying no Y was proved, in some cases at least, to be accomplished by means of paracentric gross interstitial deletions (MULLER and HERSKOWITZ 1954), it became reasonable to consider that all or almost all detachments represented multibreak occurrences rather than single breaks that had, in the case of the centric fragment, been followed by healing of the broken end. For it was possible that in such studies many detachments could have resulted either from gross deletion or from interchange between the attached-X and an autosome and yet had not had the connection between the detached X and the other chromosome material detected (MULLER and HERSKOWITZ 1954). Accordingly, two lines of investigation were decided upon at that time. The purpose of one was to determine the frequency-dosage relationship for detachment of attached-X's (HERSKOWITZ 1954), that of the other to determine how many detachments could be proved, by genetic methods, to be interchanges between the attached-X and an autosome. The latter type of interchromosomal rearrangement has been termed "half-translocation" (MULLER and HERSKOWITZ 1954; ABRAHAMSON, HERSKOWITZ, and MULLER 1954).

In cases of detachment of the half-translocation type, the attached-X is broken rather near its centromere, into a centric "*J*" portion and an acentric "*I*", and an autosome ("*A*") is broken subterminally (anywhere on chromosome IV being subterminal). The stump of the centric *J* then becomes "capped" by the telomere-bearing autosomal tip, and/or the acentric *I* becomes "captured" by attachment to the centric autosomal remainder, in substitution for the latter's tip. It seems likely that in some of the cases found both reciprocal types of recombinant chromosomes were formed but that only one of them became incorporated into the nucleus of the mature egg, whereas in other cases two of the broken pieces failed to join and were lost as acentric and dicentric isochromatids, while the other two pieces joined and became incorporated into the egg nucleus. In the case of a half-translocation with a long autosome (II or III), an egg receiving a capped *J* must, for survival of the zygote, receive a complete maternal autosome of the type that provided the cap, so that the zygote thereby becomes hyperploid for the autosomal tip. On the other hand an egg receiving an *I* that was captured by a long autosome must,

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for survival, fail to receive a complete maternal autosome of that type, and thus forms a zygote that is hypoploid for the autosomal tip.

Either condition may be genetically demonstrable if the tip includes the normal allele of a known genetic marker, for the marker could be "covered" by the hyperploidy or "uncovered" by the hypoploidy of the tip. Capturing would also be recognizable by X-autosome linkage. The hypoploidy associated with capturing would in addition give rise to a Minute-bristle phenotype in many cases.

In the interest of efficiency, the investigation of the frequency-dosage relation was carried out first, and the flies with detachments of the attached-X, thus produced, were used in the present, second line of study. These different cases of detachment were put through a series of genetic tests in order to identify cases of capping and capturing of the detached X by the autosomes. The results are discussed in relation to the problem of new telomere formation as well as to that of the factors involved in the production of gross chromosomal rearrangements in oocytes.

MATERIALS AND METHODS

The detachments tested were obtained from oocytes which contained, before treatment, a "snoc" attached-X chromosome having the genes *sc ct*^{n} *oc car* on one of the arms and *y In49 sn*^{z2} on the other (MULLER 1954). No Y chromosome was present. The detachments studied were of two kinds. One type came from an attached-X which had a breakage in or near the heterochromatin proximal to the centromere and a second subterminal breakage proximal to *sc* or *y* of the same arm, which resulted in what was in effect detachment after deletion of the interstitial piece. Such cases of detachment, bearing both *y*$^{+}$ and *sc*$^{+}$ and thereby phenotypically recognizable, served as "controls" for demonstrating that the genetic tests used to detect X-autosome half-translocations would not appear to give positive results in cases that did not actually have half-translocations. The other type of detachment, bearing either *y*$^{+}$ or *sc*$^{+}$, but not both, included whatever cases of half-translocations were viable (HERSKOWITZ and MULLER 1954; HERSKOWITZ 1954).

Detachments which were viable and fertile in the male were maintained as stocks by crossing them with attached-X females having the genotype $Y/y\,f: = ♀$. Those detachments which were lethal or incapacitating to the male were kept in heterozygous females whose other X chromosome was *y sc*^{s1} *B In49 v*. The stocks of detachments kept in the females were well balanced in those cases in which the detached X comprised the *oc*-bearing arm. In the cases having the *In49*-bearing arm there was occasional double crossing over but this did not result in the loss or unbalancing of either terminal region of the detached X, although the markers, excepting the scute S1, might become lost or change places. However, because of this situation, the possibility is not excluded that some half-translocations with the *In49* bearing arm remained unproved.

The testing procedure involved crossing flies with detachments to a series of stocks containing mutant genes located close to various autosomal termini. The mutants chosen were: *net* and *al*^{2} for IIL, *M33a* and *sp*^{2} for IIR, *ru* for IIIL, *bv* for IIIR, *spa*^{Cat} (to be referred to as *Cat*), *sv*^{n}, and *gvl* for IVR, Not only are these genes

as close to the chromosome termini as feasible, but they also have clear-cut phenotypic effects that make possible the recognition of a hyperploid condition of the given region, while at the same time this recognition is not usually prevented in cases in which there is more or less than the normal amount of heterochromatin present.

Assume, for illustration, that a fly has a detached X bearing a cap from IIR long enough to include $M33a^+$ (the normal allele of Minute 33a). Such a fly has two more $M33a^+$ loci located on the normal II chromosomes. When by means of a testcross one of the normal II chromosomes is replaced by one bearing the "haplo-insufficient" deficiency *M33a*, the Minute character will fail to show since the flies will be diploid for $M33a^+$.

The analysis with *Cat* is somewhat similar, since two doses of the normal allele, Cat^+, and one of *Cat* give a Cat^+ phenotype, and since disjunction of the free IV chromosomes is seldom prevented by the partial IV chromosome which is attached to the X. Hence, gametes containing an X capped by a Cat^+ part of IV nearly always contain a free IV, with Cat^+, in addition, if the parent from which they were derived contained two free IV's, each with Cat^+. Such gametes when united with ones (from the other parent) known to carry *Cat* result in offspring which are phenotypically Cat^+. In such a case, the identification of which offspring received *Cat* from the other parent is made possible by having this parent carry *Cat* (which is lethal when homozygous) balanced over another lethal dominant such as ci^D, which is expressed even when present in single dose in a triplo-IV fly. In contrast to this, flies derived from gametes that did not carry the capped X but carried one Cat^+-containing IV, united with *Cat*-containing gametes of the other parent, do manifest the Cat character.

The above account assumes that the fly with the capped X contains two free IV's. This will nearly always be true in later generations derived from the original exceptional individual in cases in which the Cat^+-containing cap on the X was not long enough to include the normal allele of Minute-IV. For such flies (produced originally from gametes in which a free IV had not disjoined from the part of IV capping the X) have a much higher viability and fertility and shorter developmental time than the Minute-IV's.

Even in the cases in which the Cat^+-containing cap did include $M\text{-}IV^+$, increasing numbers of the flies of later generations come to contain two free IV's, by the frequent nondisjunction whereby one free IV gets into the same gamete as the cap, since this hyperploid condition, once established, tends to be inherited in a relatively stable fashion owing to the comparative lack of influence of the cap on the segregation of the two free IV's. If however an individual with just one free IV happens to be chosen for the testcross with Cat/ci^D the result is less clear-cut than if two free IV's were present. Yet even here the presence of the cap will usually be recognizable. For this cap will undergo frequent nondisjunction with the one free IV. Thus many (up to 50%) of the offspring that receive the capped X from one parent and *Cat* from the other will appear Cat^+, while all offspring that fail to receive the capped X but do receive *Cat* will appear Cat. This will in such a case prove the presence of Cat^+ on the X.

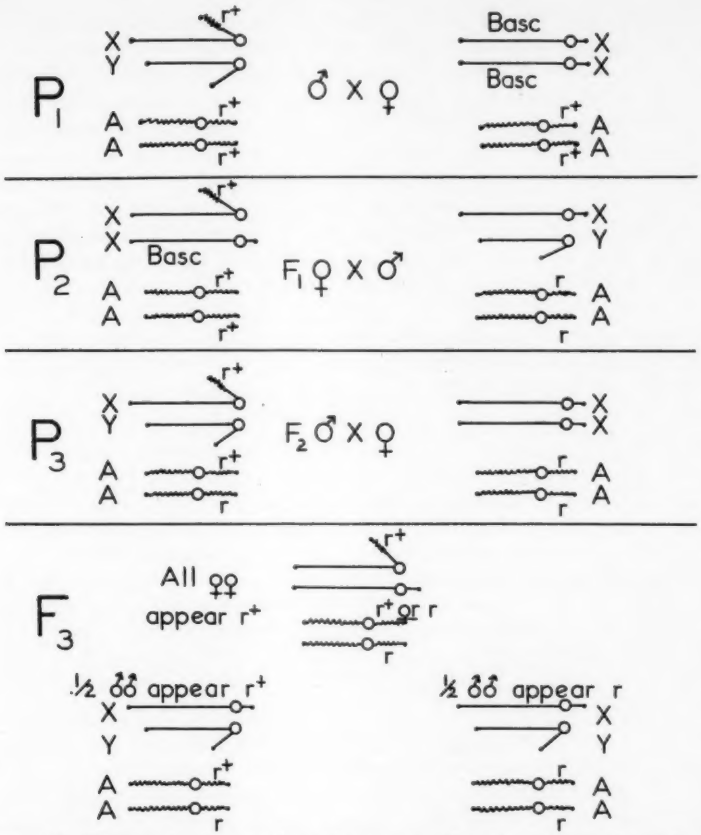

FIGURE 1.—Diagram of matings to detect the presence of an autosomal gene r^+ contained in a telomere-bearing cap which has joined to the stump of the centric arm of a broken attached-X chromosome. The X chromosome in question was present in the P_1 male. X = X chromosome carrying either *sc cl*^{n} *oc* (*car*) or *y In49 sn*^{x2} (*car*), Y = Y chromosome, A = autosome normally carrying the locus of r^+ or *r*, *Basc* = X chromosome rarely giving crossovers with a wild type X, containing *sc*^{81} *B In-S apr sc*^{8}, 0 = centromere, · = telomere. All flies are assumed to carry two complete A's in addition to the hyperploid segment capping the X (see text for discussion of another situation which applies only to chromosome IV).

If the X tested did not carry an r^+-containing cap, in the F_3, one half the females would appear r and one half r^+, the expectation for males being, however, unchanged.

If the normal allele (r^+) of a recessive marker (*r*) is present in hyperploid condition as a cap on the X it can be demonstrated as follows. When the detached X is maintained in the male (fig. 1), males are crossed to "Basc" females and the heterozygous Basc F_1 females mated to *r*/*r* males. Non-Basc (F_2) sons carry the detached X and all are therefore $r^+/r^+/r$. When these males are backcrossed to *r*/*r* females all daugh-

ters are phenotypically r^+ while one half the sons appear r^+ and the other one half r. If, on the other hand, the detachment does not carry an r^+-containing cap the offspring from the last cross would have one half of each sex appearing r^+ and the remainder r. If the cap on the X were from IV, even if the P_3 male had only one free IV instead of two, all F_3 females would still appear r^+, while all the males would in this case be r.

When the detached X is carried in the female along with *y sc*^{s1} *B In49 v* (fig. 2), these females are crossed to r/r males. If the detached X has an r^+-containing cap, present in hyperploid condition, the F_1 non-Bar females carrying the detached X are all $r^+/r^+/r$. When these F_1 are backcrossed to r/r males, three fourths of their fe-

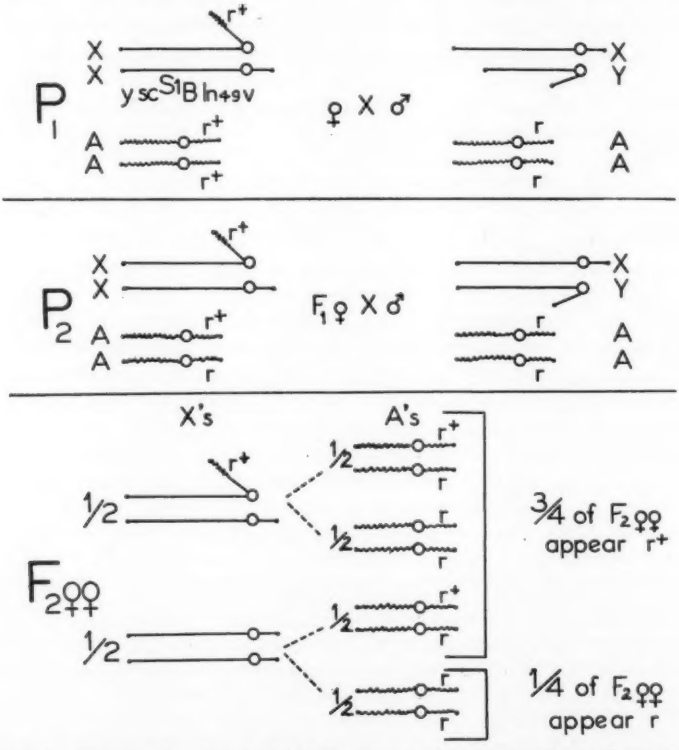

FIGURE 2.—Diagram of matings to detect the presence of an autosomal gene r^+ contained in a telomere-bearing cap which has joined to the stump of the centric arm of a broken attached-X chromosome. The X chromosome in question was presented in the P_1 female whose other X contained *y sc*^{S1} *B In49 v*; crossovers were produced rarely when the X tested carried *sc ct*^{n} *oc* (*car*) and occasionally when it carried *y In49 sn*^{s2} (*car*). The other symbols used are the same as defined in the legend to figure 1. All flies are assumed to carry two A's in addition to the hyperploid segment capping the X (see text for discussion of another situation which applies only to chromosome IV).

If the X tested did not carry an r^+-containing cap, in the F_2, one half of the females would appear r^+, the rest r.

male offspring (all of this with the detached X and half of the others) will appear r^+ and one fourth r. This is not true of the male offspring since the males with the capped X are seldom able to live. For this reason only F_2 females were scored. If, however, the detached X did not carry an r^+-containing cap one half of each sex appearing from this cross would look r^+, the rest r. Were the cap on the X derived from IV, and had the P_2 females only one free IV instead of two, one half the females and males would appear r^+, the other one half of each would appear r, and it would not be possible from these results to prove the detachment was a half-translocation. But this situation would be infrequent in view of the previous discussion and the fact that a number of P_2 females were tested in each case.

In case the detached X was of type *y In49 sn*^{z2} the Bar is, as previously explained, a poor marker but scute-S1 remains a valid marker. Hence in these cases the females of the stock having the detached X should preferably be crossed to males having both *sc* and the marker "*r*". When these are unavailable the non-Bar F_1 females should be bred individually and attention paid only to the offspring of those F_1 females which failed to produce scute (*y sc*^{s1}) sons, since these must have contained the detached X. Unfortunately this was not always carried out but the absence of scute sons was verified. It will be seen in the Discussion that from the results of these crosses and backcrosses it is possible to detect cappings or capturings involving chromosome IV and cappings by chromosomes II and III, provided one or more of the loci tested were involved in the half-translocation.

A series of additional crosses were made to detect, by means of linkage studies, cases of capturing of an acentric detached X by autosomes II or III.

All work was carried out at 25 ± 1°C.

RESULTS

Four different detachments known to result from intra-arm deficiency were used in the "control" tests for half-translocations. Three of these were maintained in the female and one in the male. Of the 20 detachments originally detected in the male and the 78 detachments originally detected in the female (HERSKOWITZ 1954) which gave y or sc phenotypes and hence possibly arose as half-translocations, 19 stocks maintained in the male and 27 stocks maintained in the female (of which only 2 were male-viable) were put through testcrosses. For 7 additional detachments the data from one or another cross are insufficient and are not presented. However, for most of the cases of detachment from which fertile stocks had been obtained fairly adequate tests were carried through.

The data for the 4 "control" and 46 "experimental" detachments, concerned with whether or not an autosomal genetic marker is included in a half-translocation in which a detached arm of "snoc" was capped or captured, are summarized in table 1. Since it was neither possible nor feasible to test all cases of detachment for all the autosomal markers used, the number of cases actually tested for a given marker is given in parenthesis under that marker in all rows in which data from more than one case are presented. The "controls" show that in a total of 30 tests for the presence of autosomal markers, including all of the 9 factors studied, there were no (false) positive cases.

Among the 46 other detachments studied 24 were proven by these tests to be half-

TABLE 1

Autosomal marker is included (+) or is not (−) in a half-translocation in which a detached arm of "snoc" was capped or captured

No. of detachments tested	L II R				L III R		IVR			Interpretation
	net$^+$	*al*$^+$	*sp*$^+$	*M33a*$^+$	*ru*$^+$	*b*$^+$	*gvl*$^+$	*sv*$^+$	*Cat*$^+$	
"Control" 4	−(1)	−(4)	−(4)	−(4)	−(4)	−(4)	−(1)	−(4)	−(4)	Gross deletion
"Experimental"*										
1	+	+	−	−	−	−	−	−	−	Capping
1		−	−	+	−			−	(−)	Capping
1	−	−	+	+		−	−	−	−	Capping
1		−	−	−	−	−	−	−	+	Capping
1	−	−	−	−	−	−	−	+	+	Capping
1	−	−	−	−	−	−	+	−	−	Capturing
18	−(2)	−(15)	−(15)	−(16)	−(15)	−(6)	+(6)	+(14)	+(17)	Capping or Capturing
22	−(11)	−(17)	−(17)	−(19)	−(16)	−(17)	−(14)	−(18)	−(20)	Unknown
— 46**										

* The numbers of *In49*-bearing detachments maintained as stocks in the female were in the different experimental rows: 1 (row 2), 11 (row 7), 6 (row 8).

** For 7 additional detachments the data are insufficient.

translocations. One involved a cap from IIL which included the loci *net*$^+$ and *al*$^+$. Two were cappings by IIR: one of these cases included the locus *M33a*$^+$ but not the more proximal *sp*$^+$, the other included both loci. Two cases of capping by IVR were demonstrated: in one the cap included *Cat*$^+$ but not the presumably more proximal *sv*$^+$, while in the other both of these factors but not the still more proximal *gvl*$^+$ factor were included. One case of capturing by IV was identified by *gvl*$^+$ being linked to the X chromosome detachment while *Cat*$^+$ and *sv*$^+$ were not. Figure 3B, C shows why this case is regarded as capturing, the preceding ones as capping. Eighteen other cases were found of X-IV half-translocations which could have been either of capping or capturing type since all the loci tested for were found present on the detachments. Here the alternatives of a capping in which IVR was broken proximally to *gvl*$^+$ or a capturing following breakage of the heterochromatic IVL arm were equally possible, as shown in figure 3D.

In no case among the "experimentals" or "controls" were there seemingly false positives that indicated the presence of two different autosomal segments on the detached X.

Most of the 22 detachments not demonstrable as half-translocations by hyperploidy of the autosomal markers used were tested for capturing by means of linkage tests between the X and the two major autosomes (table 2). None of these had appeared Minute and therefore, if cases of capturing, must have involved breaks so near the end as not to remove the locus of an *M*$^+$ gene. One of 21 stocks tested for X-II linkage gave a positive result, while none of 16 tested for X-III linkage gave a positive result.

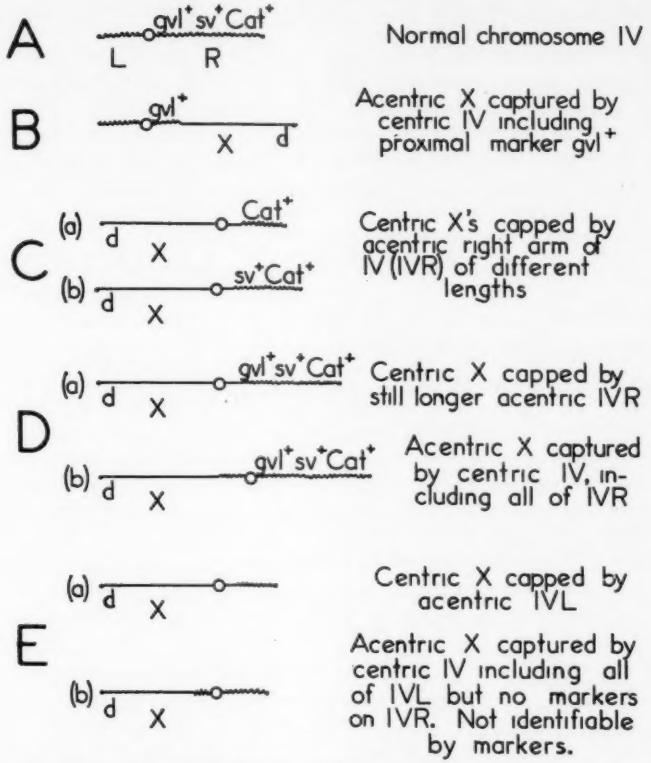

FIGURE 3.—Cappings and capturings of centric and acentric detachments of an attached-X chromosome by chromosome IV. Symbols are the same as in legend to figure 1. The major portion of the detached X is marked by "X" and its distal tip by "d".

A. Normal gene arrangement in chromosome IV (see text for evidence of order for *Cat*+ and *sv*+). B. Acentric arm of X captured by centric part of IV, proved because *gvl*+ is linked to the X while *Cat*+ and *sv*+ are not. C. Centromere-bearing X capped by accentric fragments of IV carrying, as in C(a), *Cat*+ but neither *sv*+ nor *gvl*+, or, as in C(b), *Cat*+ and *sv*+ but not *gvl*+. D(a) Cappings and D(b) capturings which are not distinguishable from one another by the markers for IV since all of these or all tested for were found linked to the X. E(a) Cappings and E(b) capturings which are not identifiable as half-translocations by means of markers since only unmarked portions of IV are joined to the X.

TABLE 2

Results of tests for linkage between X and long autosomes

Autosome tested	Positive tests	Negative tests
II	1*	20
III	0	16

* Interpretation of this case (No. 58, included among the 22 "unknowns" of table 1) is that the detached X was captured by II.

DISCUSSION

The results contain genetic proof, in support of the strong indirect evidence from X-ray frequency-dosage experiments (HERSKOWITZ 1954), that detachment of attached-X chromosomes results, in a high proportion of the cases, from chromosomal rearrangements of half-translocation type. For of the 46 detachments studied adequately 25, or about half, were demonstrably X-autosome half-translocations. The four of these with chromosome II involved 3 cappings and 1 capturing. The 21 other proved cases were interchanges with IV, among which it was possible to identify 2 cases as consisting of capping and 1 of capturing.

The identification of such a high proportion of detachments as half-translocations makes it extremely likely that the 21 unidentified detachment cases arose either as capturings by IVR broken proximally to *gvl*$^{+}$, or as cappings or intra-X deletions in which the breakage that produced the cap was distal to any of the loci tested for. In this connection three points should be especially noted. (1) The genetic markers for III are not nearly so satisfactory as those for II, being much further from the distal ends of the map; this makes it particularly likely that some cappings with III were unidentified. (2) The absence of markers for IVL, all of which is heterochromatic and probably highly breakable, would prevent detection of capping by this arm. (3) It is possible that some detached X's, especially of the *y In49 sn*^{x2} type kept in the female, failed to be identified as half-translocations because they had lost, by crossing over prior to the testcrosses, sex-linked markers used to distinguish them. It is also possible that the capping or capturing by a relatively long segment of IV would have so complicated the disjunction of free IV chromosomes that the tests to prove half-translocation were not clearly positive or negative, and thus led to the exclusion of such detachments from the data. Hence it is clear that while the half-translocations must be at least as numerous as inferred, and of the types designated, the methods used would have permitted some of those originally present to escape detection.

The present experiments, by giving direct evidence of the abundance of half-translocations, demonstrate that the exponential frequency-dosage relationship found for detachment represents multi-breakage events, as had been presumed. Without this direct evidence it might have been postulated instead that single breakages had caused the detachments but that these breaks required one or more additional "hits" for their "healing". Thus the results make less tenable the view that in Drosophila single breakages can become healed.

As expected because of the generally lower viability of hypoploids than of hyperploids, fewer cases of capturing than of capping were found. Another datum worthy of note is the relatively large number of half-translocations with IV, also found by LINDSLEY and NOVITSKI (1953) and by PARKER (1954). Several factors may operate to favor this. There is almost certainly a greater amount of breakable material, or at least a higher effective breakability, in IV than in the subtermini of other chromosomes, especially in view of the fact that viable half-translocations can result from breakage anywhere in IV, including the heterochromatic regions near the centromere. Moreover, IV in oocyte stages probably lies closer to the proximal heterochromatin of the X than do the subtermini of other chromosomes—proximity of breaks favoring interchange, especially in oocytes. Finally, for cases of capturing, the possibility exists that the size of the tip of X, II or III which is viable in hypoploid condition

may be smaller than either arm of IV, which is viable (as is the whole of IV) in haploid condition. This argument acquires cogency when we consider not total inviability but the relative inviability associated with Minute deficiencies, inasmuch as breaks very near the termini of II and III would result in Minute hypoploids, whereas no break in IVL and only breaks in a relatively small proximal portion of IVR would give a Minute hypoploid. The corresponding argument relating to viability in cases of cappings is probably invalid, at least for some of the distal pieces in question, since some viable hyperploids for tips of II and III are known with more extra material than in IV.

The case of capping by IVR in which the cap contained Cat^+ but not sv^+ nor gvl^+ is evidence that Cat^+ is more distally located on IVR than these other two factors.

SUMMARY

It has been possible to prove genetically that at least one half of the tested cases of detachment of attached-X chromosomes by X-rays consisted of half-translocations with autosomes which involved sections of chromosome long enough to contain an allele of a known genetic marker, yet short enough to allow survival. Both cases of capping and of capturing were identified but (by the conditions of the testing) more of the former.

While it is known that recoverable cases of detachment ordinarily require more than one event for their occurrence (HERSKOWITZ and MULLER 1953; HERSKOWITZ 1954; PARKER 1953; KUTSCHERA 1954), it is proved here that these events involve breakages in different chromosome regions, which then undergo interchange of pieces. The alternative postulate, that there are two events at a single chromosome site, namely, breakage and then healing, each requiring a separate hit (or at least the combination requiring two hits) is thereby rendered improbable.

An excess of X-IV half-translocations was found. Possible explanations suggested for this excess are differences in distribution of chromatin, especially heterochromatin, in viability effects of aneuploidy, and in proximity of breakages.

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INDUCED CHANGES IN FEMALE GERM CELLS OF DROSOPHILA. I. DEPENDENCE OF HALF-TRANSLOCATION FREQUENCY UPON X-RAY DELIVERY RATE[1]

IRWIN H. HERSKOWITZ AND S. ABRAHAMSON[2]

Department of Zoology,[3] Indiana University, Bloomington, Indiana

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IT HAS BEEN definitely established for X-ray-induced mutations in plants that lowering the intensity of treatment causes a decrease in the number of chromosomal rearrangements obtained (SAX 1939a,b, 1940, 1941; SAX and BRUMFIELD 1943). This observation could result from either or both a necessity for several "units" of X-rays to accumulate in order to produce a break (and as the rate of delivery decreases some units are lost before others are produced), and a decrease in rejoinability of breaks with time (by the restitution of some breaks before others are produced, and/or by the movement of broken ends so far apart from others that their chance of ever combining is reduced). Study of Drosophila sperm for gross rearrangement frequency after X-ray treatment of inseminated females revealed no effect of extremely different rates of treatment (MULLER 1938, 1939a,b, 1940; RAY-CHAUDHURI 1939, 1944; MAKHIJANI 1945), supporting none of the hypotheses mentioned for sperm known to be mature. Recently, HAAS, DUDGEON, CLAYTON and STONE (1954), using delivery rates higher than those used earlier, have reported that when sperm were treated in males the higher rate of delivery gave more translocations than the lower rate. One would want this intensity effect repeated with samples of sperm of greater uniformity, for example with sperm treated in inseminated females, however, before accepting it without reservation, since it has been shown that the translocation frequency from sperm delivered by treated males on one day can be twice what it is on a successive day (and possibly in successive copulations on the same day) (ABRAHAMSON and TELFER 1954).

While a study of breakage dependence upon X-ray intensity can decide whether break production requires the accumulation of "units", and the best evidence is that no such accumulation is required for breakage itself, a study of restitution and rearrangement can tell whether breaks are temporarily unjoinable and accumulate until a specific time or whether broken ends can join to other broken ends at any time. The infrequency of gross deficiencies and inversions and of reciprocal translocations in the germ cells of female Drosophila has prevented the latter type of study in this organism until recently, although MULLER, VALENCIA and VALENCIA (1949) inferred union between fragments to occur fairly promptly, i.e. prior to the movements of the meiotic divisions. Such a study became possible once it was shown (HERSKOWITZ and MULLER 1953; MULLER and HERSKOWITZ 1954; HERSKOWITZ

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[2] Eigenmann Fellow in Zoology.

[3] Contribution No. 611

1954a,b; ABRAHAMSON, HERSKOWITZ and MULLER 1954, 1956; PARKER 1954a,b) that a certain type of multi-break rearrangement does occur frequently in oocytes after X-ray treatment. This type of mutation was termed a "half-translocation" because it involved, of the four pieces produced by breaking two non-homologous chromosomes, only two pieces which joined eucentrically (making up one half of a reciprocal translocation) and were retained in the fertilized egg, the other centric piece having been cast into a polar body joined or unjoined (what the situation usually is has not yet been proven) to the other acentric fragment.

The type of half-translocation studied here was one in which an attached-X chromosome was broken into two arms, only one of which was retained in the egg after joining eucentrically to a piece of another broken chromosome, the other, reciprocal, pieces going into a polar body. All the present experiments were designed to detect whether there was any difference in percentage of offspring with detached-X's (detected whenever these became viable half-translocations) following different X-ray delivery rates. Part of the results presented here were reported by HERSKOWITZ and ABRAHAMSON (1955).

MATERIAL AND METHODS

Using *Drosophila melanogaster*, virgin females were obtained, carrying the "snoc" attached-X chromosome (*sc ct*^{n} *oc ptg car·sn*^{z2} *In49 y*) and either no Y chromosome or a modified Y (*y*$^{+}$ *sc*^{v1}·Y^{s}), and were aged for at least 3 days under excellent nutritional conditions. The virgins were then irradiated and mated in one half pint bottles to males carrying an X (*y sc*^{s1} *B In49 v*) and a normal Y chromosome. When 1000r were delivered the number of parents per bottle was 30♀♀ and 60♂♂; for treatments of (or about) 2000r, the number of parents per bottle was approximately doubled. From eggs oviposited within the first 4 days after the completion of irradiation (the parents having been transferred to fresh food bottles once or twice within this period, except in Experiment I where eggs deposited only during the first two days after treatment were used), the F_1 flies were scored for detached-X half-translocations. Whether or not the irradiated mothers carried the modified Y chromosome, daughters carrying detached-X half-translocations were phenotypically non-vermilion and "heterozygous" Bar-eyed while triplo-X daughters also would be non-*v* and (wide) Bar, but sterile. The unexceptional daughters were round-eyed. Occasionally, by paternal nondisjunction, vermilion "homozygous" Bar-eyed females were produced. F_1 females due to paternal nondisjunction are not included in the tables. Sons carrying a detached-X were round-eyed, whereas unexceptional sons were vermilion and Bar-eyed (and, incidentally, sterile). The irradiations were administered to non-narcotized, well-aerated flies, at a peak kilovoltage of 200 and at 20 ma through a 1 mm thick Al filter; a Victoreen dosimeter was used to check the machine's output, just before the first and sometimes also between and after treatments in each experiment. The delivery rate to the flies was varied either by changing the target distance or by stopping the X-ray machine for different periods of time before the total dose was delivered, or both. The details of these procedures are given with the results of the specific experiments.

In experiments where the dilute treatments required more than 8 hours to de-

TABLE 1

Percentages of half-translocations in the F_1 from different experiments. (The totals of F_1 scored are given in parentheses. The exposure factors for Y-less mothers were identical to those for the corresponding treatment for Y-bearers in the same experiment.)

Experiment	Total dose	Modified Y-bearing mothers		Y-less mothers	
		Concentrated	Dispersed or dilute	Concentrated	Dispersed or dilute
I	2000r	600r/min continuously	30r/min in 2 parts 8 hrs. apart		
		5.4 (336)	1.7 (534)		
II	2000r	600r/min continuously	600r/min in 4 parts 8 hrs. apart		
		1.9 (1935)	0.9 (2836)	1.3 (549)	0.8 (877)
III	1000r	1100r/min continuously	30r/min continuously		
		1.6 (2905)	0.7 (3306)	1.2 (1567)	0.5 (1477)
Controls		0.05 (2025)		0 (691)	

liver, all the treated flies for that experiment were handled alike, in special plastic containers, one side of which was covered with dacron netting, which could be placed on food to permit the virgins to feed between the parts of the irradiation procedure.

All experiments were carried out at room temperature.

RESULTS AND DISCUSSION OF INDIVIDUAL EXPERIMENTS

In the first type of experiment (Exp. I, table 1) a total dose of 2000r was given either continuously at 600r/min. (taking only 3′20″ for delivery) or at 30r/min. in 2 parts 8 hours apart (requiring 33′20″ to deliver each of the two parts). Thus the females subjected to the dilute treatment had X-rays delivered at a rate 20× slower than females given the intense treatment and had in addition an intermission of 8 hours midway in the irradiation. The half-translocation rate obtained was 5.4% for the intense and 1.5% for the dilute treatments ($P < .005$) in oocytes of the modified Y-bearing females. This result proves that the breaks produced in oocytes which enter into gross rearrangements do not always remain unjoined, as they do in the mature sperm (until after fertilization), for 8 hours after being produced. The amount of decrease in half-translocation frequency with protracted treatment permits the estimation that about two thirds of the breaks which, in the intense treatment, were present in cells having a second break with which they half-translocated, were, in the protracted treatment, in cells where this second break had already either restituted or become "unjoinable".

In the second type of experiment (Exp. II, table 1) 2000r were again delivered, in the intense treatment continuously at 600r/min., as in Exp. I, and in the "dilute" also at 600r/min. but in 4 equal parts 8 hours apart. The intense and dilute percentages were respectively 1.9 and 0.9 for the modified Y-bearing females ($P <$ .005). In this experiment, since the rate of output of the X-ray machine was the same in the contrasting treatments, the difference noted must be due to breaks becoming unavailable for interchange in the interval between the successive irradia-

tions. If in the dilute treatment all breaks produced in the first one quarter of the irradiation which were ever to restitute or rearrange did so before any breaks in the second one quarter of the treatment were produced, etc., then the total half-translocation frequency for the dilute would equal 4× the rate induced by one quarter of the total dose. Since it was found (HERSKOWITZ 1954b) that half-translocations increase, in this dose range, as the $\frac{3}{2}$ power of the dose (however, see General Discussion also), then for "total dose/4" one would expect $\frac{1}{8}$ the half-translocations produced by the total dose, and since there were 4 such one quarter doses given in the dilute one would expect from the dilute $4 \times \frac{1}{8}$ or $\frac{1}{2}$ the number of half-translocations obtained from the intense treatment. However, the rate for the dilute would be more than $\frac{1}{2}$ that for the intense if there were an appreciable number of breaks open and rejoinable 8 hours after treatment. The mutation percentage obtained for the dilute being just about one half that for the intense strongly supports the view that almost all if not all breakages which are ever going to join do so within 8 hours following treatment.

Because the percentage for the dilute treatment in Experiment I is less than $\frac{1}{2}$ the rate of the intense (actually 1/3.6, with treatment having been given in only two parts 8 hours apart), there is good reason to believe that breaks were becoming unavailable for interchange during the extra 63′20″ the X-ray machine was operating in the dilute treatment. Thus the first two experiments strongly suggest that within an hour after breaks are produced an appreciable number of broken pieces can restitute or interchange and that if they are to do either they must do so within 8 hours of their production.

The third experiment (Exp. III, table 1) required giving 1000r in continuous treatments either at a rate of 1100r/min. (54″ delivery time) or at 30r/min. (33′20″ delivery time). The incidences of half-translocations for intense and dilute treatments were, respectively, 1.6% and 0.7% for modified Y-bearing mothers ($P < .005$). Since an average of about 16 minutes elapsed between the production of successive breaks in the dilute treatments but averaged less than one half minute in the intense, the reduction in half-translocation percentage for the dilute to about one half the value for the intense is strong evidence that under these experimental conditions at least one half the breaks that are to undergo restitution or interchange do so within about 16 minutes of their production.

Whenever, as in Experiments II and III, Y-less females were also treated at the times modified Y bearers were, a drop in half-translocation frequency with dosage dilution was obtained which was similar in magnitude to that for modified Y bearers. Although this drop was not statistically significant, its indecisiveness was probably caused only by its small sample size.

The purpose of a fourth experiment was to determine whether evidence could be obtained for a change in joinability of breaks in periods of varying lengths. To do this it was decided to deliver a total dose of 2200r at the rate of 1100r/min. in 8 equal periods with 0, $\frac{1}{4}$, $\frac{1}{2}$, 1, 4, 16, or 32 minutes between successive irradiations. (For example, the total period covered by the treatment when 1 min. intervals were used was 9 minutes.) In accord with the preceding discussion it can be reckoned that at this total dose the half-translocation rate for the most dispersed treatment in this

TABLE 2

Percentages of half-translocations produced by 2200r delivered at 1100r/min. in 8 equal periods with the various time intervals indicated between successive exposures. (The totals of F_1 scored are given in parentheses.)

Minutes between exposures	Total period of treatment	Brood 1	Brood 2	Brood 3	Total
0	2 min.	4.5 (198)	8.5 (658)	5.1 (938)	6.3 (1794)
¼	3¾ min.	9.9 (132)	5.2 (232)	7.3 (359)	7.1 (723)
½	5½ min.	8.0 (88)	6.7 (233)	5.9 (405)	7.3 (726)
1	9 min.	8.4 (95)	8.4 (220)	7.0 (366)	7.6 (681)
2	16 min.	4.9 (61)	11.2 (187)	6.0 (317)	7.6 (565)
4	30 min.	6.4 (109)	8.9 (273)	7.1 (383)	7.6 (765)
Σ0–4		6.9 (683)	8.8 (1803)	6.1 (2768)	7.1±0.35 (5254)
16	1 hr. 54 min.	1.9 (54)	6.2 (517)	4.8 (645)	5.3±0.64 (1216)
32	3¾ hrs.	1.9 (158)	4.7 (796)	2.7 (1020)	3.4±0.41 (1974)
Unirradiated (Control)		0 (202)	0 (1318)	0 (661)	0 (2181)

experiment can be as little as (but probably no less than) 1/3.5 of the rate for the most intense treatment, at which there was no interval between the irradiations, provided that the breaks produced in different one eighths of the most dispersed irradiation are unable to join with each other. The shorter the intervals between irradiations the more likely it would become that some breaks from earlier one eighths would still be available for rearrangement with breaks produced in later one eighths, causing the percentage of detachments obtained to approach that for the most intense treatment. The results from eggs oviposited the first day after irradiation, the next two days, and the fourth day, are presented as broods 1, 2, and 3, respectively, separately and also combined, in table 2. As expected, the intervals of 32 minutes between irradiations gave half-translocation percentages which were lower than the values in corresponding broods for all other, less dispersed treatments, in 20 of 21 possible comparisons, although they were equal in value in the 21st. Also as expected, the 16-min. intervals between irradiations gave half-translocation percentages which were lower than the values in corresponding broods for other, less dispersed treatments in 17 of 18 possible comparisons. This might be taken to mean that more unjoined but joinable breaks overlapped the different fractions of the treatment in the 16-min. than in the 32-min. intervals. However, for the 4-min. intervals, in which the total treatment period covered 30 minutes, the values were lower than in corresponding broods of less dispersed treatments in only 5 of 15 possible comparisons. It is clear from the frequencies for different broods separately and combined that no difference has been detected between the treatments having intervals in the range of 0–4 minutes. The half-translocation rate when the periods between irradiations were 4 minutes or less (the treatments requiring 30 minutes or less) averaged $7.1 \pm 0.35\%$. They decreased from this value to $5.3 \pm 0.64\%$ ($P = .025$) with the 16-min. inter-

vals (where the treatment took almost 2 hours) and to $3.4 \pm 0.14\%$ ($P < .0001$) with the 32-min. intervals (which required nearly 4 hours to deliver). The frequency for the 32-min. intervals is only ½ that for intervals of 0–4 minutes. However, since it appears significantly higher than 1/3.5 of the latter value, it is probable that, under these latter experimental conditions, some breaks that are produced 32 or more minutes apart cooperate to produce half-translocations, although most of the breakages which join do so within 4 hours of their production.

GENERAL DISCUSSION

The present establishment that the number of detached-X half-translocations depends upon the amount of dispersal of the X-ray dose, is at the same time an independent demonstration that half-translocations have an origin that is multi-event and not single (such as the production of a break which is at the same time "healed" to form a new telomere), supporting the other evidences for multi-event origin obtained from X-ray dosage studies (HERSKOWITZ and MULLER 1953; HERSKOWITZ 1954a,b; and, independently, PARKER 1954a) and from genetic studies for identification of half-translocations (ABRAHAMSON, HERSKOWITZ and MULLER 1954, 1956; PARKER 1954b).

The higher rate of half-translocation in oocytes containing the modified Y chromosome ($y^+ sc^{v1} \cdot Y^s$) than in Y-less oocytes, discovered by PARKER (1954b), has been confirmed in all 4 possible comparisons in the present experiments (table 1). At least a part of the reason for this difference, it may be proposed here, lies in the fact that, in an oocyte, where the chromosomes are assumed to be in tetrad condition, when a half-translocation between the X and an autosome enters the mature egg there is about one chance in four that the reciprocal centromere-bearing piece will also enter, thereby preventing the detection of a viable half-translocation either because it produces a full translocation or (when this centromere-bearing piece has not joined to an acentric fragment) death due to dicentric formation. However, when a half-translocation between the X and Y enters the egg the reciprocal, homologous, centromere-bearing piece will almost always enter a polar body since homologous centromeres disjoin during chromosome reduction.

It should be noted that among the possible sources of heterogeneity in different experiments, other than the irradiation itself, are variations in daily temperature, humidity, nutrition, and age of the females used, requiring one to proceed with caution when comparing results of different experiments. All of these factors were carefully randomized, however, within each experiment.

A possible source of error in some experiments lies in the production of triplo-X females that might be mistaken for cases of half-translocation. In the controls there was only one detachment, a male of type *sc* cl^n *oc car*, obtained among a total of 4897 offspring, and no exceptional females, hence no triplo-X's. Since culture conditions for the treated flies were made as similar to the controls as possible (food, temperature, and crowding among developing offspring), it is extremely likely that few or no triplo-X female offspring survived in the treated cultures either. In support of this is the fact that when exceptional females of y^+ sc^+ "heterozygous" Bar phenotype, which would include all cases of triplo-X, were subtracted from the exceptional off-

spring of all irradiated series, in no case did the direction of change, in one treatment as compared with another, differ from what is given in the tables, or produce a change which would require modification of the conclusions drawn from the results as presented. For these reasons it is concluded that the number of triplo-X flies scored erroneously as half-translocations is negligible. Accordingly we have not listed separately in the tables the phenotypes, as regards *y*, *sc* or sex, of the exceptional flies.

Despite the fact that only the eggs oviposited within the first 4 days after the completion of treatment were used in these experiments, and all were "oocytes" when treated, there was a significant decrease in mutability in successive eggs oviposited. Analysis of the data shows that in every case in Experiments II and III, as was found also in earlier work (see table 1 in HERSKOWITZ 1954b), the eggs laid the first days after treatment have more mutations in them than those oviposited soon after. This is undoubtedly partially responsible for the mutation rate with the continuous treatment in Experiment II, where eggs from all 4 days were used, being lower than the rate with the same treatment in Experiment I, where only eggs from the first 2 days were used. In Experiment IV, while the mutation rate in brood 2 is lower than the rate in brood 1 in only 2 of 7 possible comparisons (probably due to chance variation in the percentages of the smaller sample sizes in brood 1), there was a lower mutation rate in brood 4 in 7 of 8 cases both when compared to the rates for brood 2 alone and for broods 1 and 2 combined.

The dilute treatment in Experiment III (table 1) and the treatment having intervals of 4 minutes in Experiment IV (table 2) gave contradictory results; whereas both were delivered in about the same period of time (33′20″ and 30′, respectively) the former showed a significant decrease in mutation rate from the corresponding concentrated treatment but the latter showed no decrease. In other words, the former case had a considerable amount of rejoining during the treatment period, while the latter had little or none. There are, however, two differences between the conditions of irradiation in these experiments, either or both of which may be responsible for the results. One difference is in the dose administered (one being about twice the other), the other is in the rate at which the X-rays were applied to the cells (slow and continuous in the former experiment and quick and discontinuous in the latter). WOLFF and ATWOOD (1954) obtained similar results in experiments with Vicia, where a lower dose produced fewer rearrangements when given at a lower rate than when given at a higher rate, but a higher dose gave the same number of rearrangements for both intense and dilute treatments. The basis for such results is still undetermined.

Since the relationship of half-translocation rate to X-ray dosage (HERSKOWITZ 1954b) was studied before the effect of concentration was known, it is desirable to re-examine this dosage relationship. The purpose of that earlier work was to determine whether one event (break) or more was required to produce the mutant type studied, to be decided from the mutant's rate of increase with dose. Whereas in the case of mature sperm there is strong reason for believing that during irradiation all breaks remain open and equally joinable (so far as those breaks are concerned which are later to rejoin), so that the time required to deliver the dose makes no difference, it has been established in the present study that, in oocytes, there can be a substantial decrease in breaks available for joining during the course of an irradiation. In the

earlier dosage study the intensity was about 250r/min. If the higher dose used (4000r), delivered in 16 minutes, did not prevent rejoining before the irradiation was completed then the exponent (observed to be the 3/2 power of the dose) was correctly estimated, but if the higher dose prevented rejoining while the lower dose (1000r) did not, or did so to a lesser degree, the exponent was an overestimation if taken as representing the relation of rearrangement frequency to breakage frequency. Which of these situations obtained is unknown at present.

A significant observation is that while the numbers of parent females irradiated at the different intensities in Experiment IV were very nearly equal (except that for the 0 min. intervals twice this number was used) the total number of offspring obtained increased in the 16 min. as compared with the 0–4 min., and in the 32 min. as compared with the 16 min. intervals. It is also noteworthy that the dispersed or dilute has more offspring than its corresponding concentrated treatment in every case in table 1 where the numbers of parents used were the same for both treatments within an experiment (Experiment III is to be excluded, however, since more parents were used in the dilute). This statistically significant increase in fecundity with dilution of the dose has been a subject for further investigation and will be reported in the next paper of this series (ABRAHAMSON and HERSKOWITZ, in manuscript).

SUMMARY

The frequency of half-translocations obtained following detachment of attached-X chromosomes was employed as the measure of multi-break chromosomal rearrangements induced by X-rays administered at various concentrations to *Drosophila melanogaster* oocytes. It was found that higher concentrations produced more half-translocations than lower ones, proving that breaks in oocytes can join in new arrangements before fertilization, unlike what is the case for breaks in fully mature spermatozoa (those in inseminated females).

The results show that of the joinings which will take place the great majority do so within 8 hours, and that about one half do so within 4 hours. Under certain experimental conditions (as in Exp. III, see p. 423) at least one half the breaks that join can do so within ¼ hour, but under other conditions (Exp. IV, see pp. 423–425) joinings in this period were not detected.

The relation these results have to earlier work on the dosage-frequency relationship for half-translocations and in confirming the influence of a modified Y chromosome on increasing their frequency is discussed.

It was found again that among oocytes oviposited during the first four days following treatment there were significantly more mutations in the eggs laid in the first days than in those oviposited in later ones. There was also a significant decrease in fecundity of the parent females in this 4-day period when the treatments were more concentrated.

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THE EFFECTS OF GENOTYPE AND CELL ENVIRONMENT ON MELANOBLAST DIFFERENTIATION IN THE HOUSE MOUSE[1]

CLEMENT L. MARKERT AND WILLYS K. SILVERS[2]

Department of Zoology, University of Michigan and Department of Biology, Brown University

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THE FOUNDATION of all embryonic development consists of the processes by which cells become differentiated from each other and from their common progenitors. These processes of cellular differentiation, though basic to an understanding of embryogeny, remain largely unknown, although two major factors are generally recognized as important in governing the course of cell development. First, the genetic makeup of cells defines the limits and the potentialities of their development, and second, the diverse cellular environments of the embryo elicit the specific developmental responses in cells which lead to their maturation into the wide variety of cell types that characterizes the adult. These local and highly specific cell environments are produced by cellular activity and are constantly changing as the responsible cells change. Thus a continually evolving dynamic interchange between embryonic cells directs their differentiation into specialized adult cells and finally into senescent cells incapable of sustaining the life of the organism.

The pigmented cells of the body—the melanocytes—have long been favored types of cells for studies of the genetic and environmental factors involved in cell differentiation. A dramatic and easily studied type of specialization characterizes these cells. Furthermore the differentiation of melanoblasts exhibits great sensitivity to changes in genetic constitution and to variations in the cellular environment in which they are transformed into melanocytes. The fact that melanoblasts can be successfully transplanted to new tissue environments has also made possible experimental analyses of the relative roles of genotype and cell environment in directing melanoblast differentiation. On the basis of previous work and that to be reported here it is apparent that within any single animal at least four distinct factors are important in melanoblast differentiation: (1) the genotype of the melanoblast, (2) the genotype of the environmental cells, (3) the environmental history of the melanoblast, and (4) the differentiated characteristics of the environmental cells—that is, whether the cells are epidermal cells, dermal cells, harderian gland cells, etc.

In this report these four factors have been considered in the differentiation of the melanocytes in house mice (*Mus musculus*) of 50 different genotypes involving genes at 15 distinct loci. The objectives of this investigation are (1) to describe the occurrence, abundance, and characteristics of melanocytes in the tissues of the nictitans, harderian gland, ear skin, hair follicle, choroid, and retina; (2) to relate these char-

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[2] U. S. Public Health Service Research Fellow of the National Institutes of Health.

TABLE 1[1]

The occurrence of melanocytes in six tissues of the house mouse

Genotype	Retina	Choroid	Hair follicle	Ear skin	Harderian gland	Nictitans
AA	+	+	+	+	+	+
AAbb	+	+	+	+	+	+
$A^{v}a$	+	+	+	−	+	+
$A^{v}abb$	+	+	+	−	+	+
$A^{1}A^{1}$	+	+	+	+	+	+
$a^{t}a^{t}$	+	NE	+	+	+	+
$a^{t}a^{t}bb$	+	NE	+	+	+	+
aa	+	+	+	+	+	+
$aaB^{lt}B^{lt}$	+	+	+	+	+	+
aabb	+	+	+	+	+	+
$aabbc^{ch}c^{ch}$	+	NE	+	+	+	+
$AAc^{e}c^{e}$	+	NE	+	+	+	+
$AAbbc^{e}c^{e}$	+	NE	+	NE	+	NE
$A^{v}ac^{e}c^{e}$	+	NE	0	0−	+	−
$aac^{e}c^{e}$	+	NE	+	+	+	+
AAcc	0	0	0	0	0	0
$A^{v}add$	+	NE	+	−	+	+−
aadd	+	NE	+	+	+	+
aabbdd	+	NE	+	+	+	+
aabbddln ln	+	NE	+	+	+	−+
aabbln ln	+	NE	+	+	+	+−
aapp	−	−	+	0	0	0
aappdd	−	NE	+	0−	0	−
aabbppdd	−	NE	+	0−	0	0−
AASl sl	+	+	+	+	−	+
aaSl sl	+	NE	+	+	−	+
$AASl\ slW^{v}w$	+	+	+	+	0−	0−
$aaSl\ slW^{v}w$	+	+	+	+	0−	0−
$aaW^{v}w$	+	+	+	+	+	+
$aaW^{v}W^{v}$	+	0	0	0−	0−	0
$aaW^{v}W$	+	0	0	0	0	0
aaWW	+	0	0	0	0	0
aaWw	+	+	+	+	+	+
AAWwss	+	0	+	+	0	0
aaWwss	+	0	+	+	0	0
$A^{v}ass$	+	+	+	−	+	+
$A^{v}abbss$	+	+	+	−	+	+
$A^{1}A^{1}ss$	+	+	+	+	+	+
aass	+	+	+	+	+	+
aabbss	+	+	+	+	+	+
aaTo to	+	NE	+	+	+	+
$aaMi^{wh}\ Mi$	+	0	+	+	0	+−
$a^{t}a^{t}Mi^{wh}\ Mi$	+	0	+	+	0	+−
$aaMi^{wh}\ Mi^{wh}$	−	0	0	0	0	0
$A^{1}A^{1}gl\ gl$	+	NE	+	+	+	+

TABLE 1—*Continued*

Genotype	Retina	Choroid	Hair follicle	Ear skin	Harderian gland	Nictitans
aaru ru	−	+	+	0−	−	−
aatp tp	+	NE	+	+	+	−
aaVa va	+	NE	+	+	+	0
aabbVa va	+	+	+	+	+	0
aabbddppey-1 ey-1 ey-2 ey-2			+	0	0	0

[1] The symbols used in this table have the following significance: + = substantially normal occurrence; − = greatly reduced number of melanocytes; 0 = no melanocytes; NE = tissue not examined. Spotted tissues are recorded as + when numerous melanocytes are present in the pigmented areas even though the white areas contain no melanocytes. Hair follicles are classified into only two types: + = pigmented, 0 = white. Although an entry is made for the retina of each genotype, an examination of sectioned eyes was made only for those genotypes in which a positive entry is also recorded for the choroid. Entries for the retinas of unsectioned eyes are based on gross observation alone. The $c^{e}c^{e}$ genotypes have numerous melanocytes but their granules are lighter than normal.

acteristics to the embryonic history and genotype of the melanocyte and to the genotype and differentiated characteristics of the surrounding cells; (3) to formulate an interpretation of melanocyte differentiation based upon gene action within the melanocyte and within the environmental cells.

MATERIALS AND METHODS

The mouse produces two types of melanocytes: (1) the dendritic melanocyte which arises from the neural crest and migrates into many tissues, in some of which it differentiates, and (2) the epidermal melanocyte which arises from the outer layer of the optic cup and which forms the pigmented layer of the retina. These two types of melanocytes were studied in 50 different genotypes of house mice—largely derived from inbred strains maintained at the Jackson Memorial Laboratory. The genotypes that were examined are listed in table 1. A discussion of some of the effects of most of these genes can be found in *The Genetics of the Mouse* by GRÜNEBERG (1952). The melanocytes and surrounding tissues were subjected to microscopic examination both in living preparations and in fixed preparations which were made as whole mounts or sections both stained and unstained.

Various tissues of the mouse were also dissected out and supravitally stained with brilliant cresyl blue in an effort to identify pigmentless melanocytes or melanoblasts that might be present in the tissue. The brilliant cresyl blue stain was used according to the procedure outlined by REYNOLDS (1954). These tissues were also incubated with dopa to reveal the presence of dopa positive cells. Incubation with dopa was for 24 hours in a hanging drop preparation containing 0.5 mg dopa/ml of M/10 phosphate buffer at a pH of 6.8.

Melanocytes are found in a wide variety of mouse tissues. They generally occur in the epidermal hair bulbs and in the dermis of the ear, tail, feet, scrotum, genital papilla, and eyelid; they also appear in the meninges of the brain, particularly between the olfactory and cerebral hemispheres, in the parathyroid, thymus, and

harderian glands, and in the nictitans, choroid, and retina of the eye. For the present investigation attention was centered on six tissues: hair bulbs, ear skin, harderian gland, nictitans, retina, and choroid. In many mouse genotypes these six tissues are abundantly supplied with melanocytes, and in nearly all the genotypes studied the characteristics and abundance of the melanocytes in each of these tissues showed a high order of constancy.

These six tissues were chosen not only because of the regularity of their behavior in influencing the differentiation of melanocytes but also because they appeared to represent very different kinds of differentiated tissue with little in common beyond the fact that they supported the differentiation of melanocytes.

The hair bulbs are composed of dense aggregations of epidermal cells that arise from invaginated thick placodes formed by the germinative stratum of the epidermis. The papilla of mesenchymal origin that pushes into the distal end of the hair bulb is important in the growth and nutrition of the hair and perhaps serves as a portal for the entrance of melanoblasts into the epidermal parts of the hair, but the papilla, itself, is unable to stimulate melanoblast differentiation. Mature melanocytes are found only among the epidermal cells in hair bulbs (fig. 1).

The dermis is formed early in embryonic life from loose aggregations of mesenchyme that migrate outward from the mesoderm to lie immediately beneath the epidermis. Here the mesenchyme differentiates into a dense connective tissue through which melanoblasts must migrate but in which they generally fail to differentiate. However, the dermis in a few limited areas such as in the ear and tail does provide an environment that induces melanoblast differentiation.

The harderian gland is of epidermal origin and arises as a solid ingrowth of cells at the inner angle of the nictitans. This gland is located in the orbit behind the eye and secretes an oily fluid that bathes the front of the eyeball. In addition the gland contains variable amounts of porphyrin depending upon the age, sex, and genotype of the mouse (STRONG 1942; BITTNER and WATSON 1946). The porphyrin characteristically occurs as reddish-brown clumps of pigment in the alveolar lumina (COHN 1955). The harderian gland in most genotypes is abundantly supplied with dendritic melanocytes distributed throughout the connective tissue that encapsulates the gland and divides it into lobules (figs. 2, 3, and 4).

The nictitans—the rudimentary third eyelid of the mouse—arises as an epidermal fold enclosing mesenchyme. The mesenchyme develops into a central core of cartilage surrounded by dense connective tissue which lies immediately beneath the two sheets of stratified epidermis. Within this epidermis the melanocytes are packed together so as to resemble an epithelium that covers both sides of the distal edge of the nictitans (figs. 5 and 6). The concentration of melanocytes sharply diminishes proximally, and the base of the nictitans is usually devoid of pigment although melanocytes can frequently be found in the adjacent connective tissue of the orbit.

The choroid of the eye lies immediately adjacent to the pigmented retina and is derived from the mesenchymal investment of the optic cup. Melanoblasts migrate into this heavily vascularized soft connective tissue layer and there differentiate into densely packed melanocytes.

The pigmented choroidal coat of the eye can readily be distinguished from the

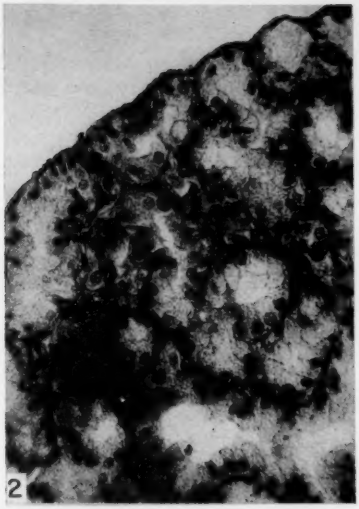


FIGURE 1.—Cleared, unstained preparation of a developing hair in the skin of a black mouse (*aa*). Note the absence of melanocytes from the region of the papilla. Melanocytes occur only among the epidermal cells of the hair. × 650.

FIGURE 2.—Cross section through the harderian gland of a black mouse (*aa*). Note melanocytes in the connective tissue between the alveoli of the gland. × 290.

FIGURE 3.—Whole mount of the harderian gland of an albino mouse at ten days of age; contrast with figure 4. × 20.

FIGURE 4.—Whole mount of the harderian gland of a black mouse (*aa*) at ten days of age. Note the abundant melanocytes scattered over the surface of the gland; contrast with figure 3. × 16.

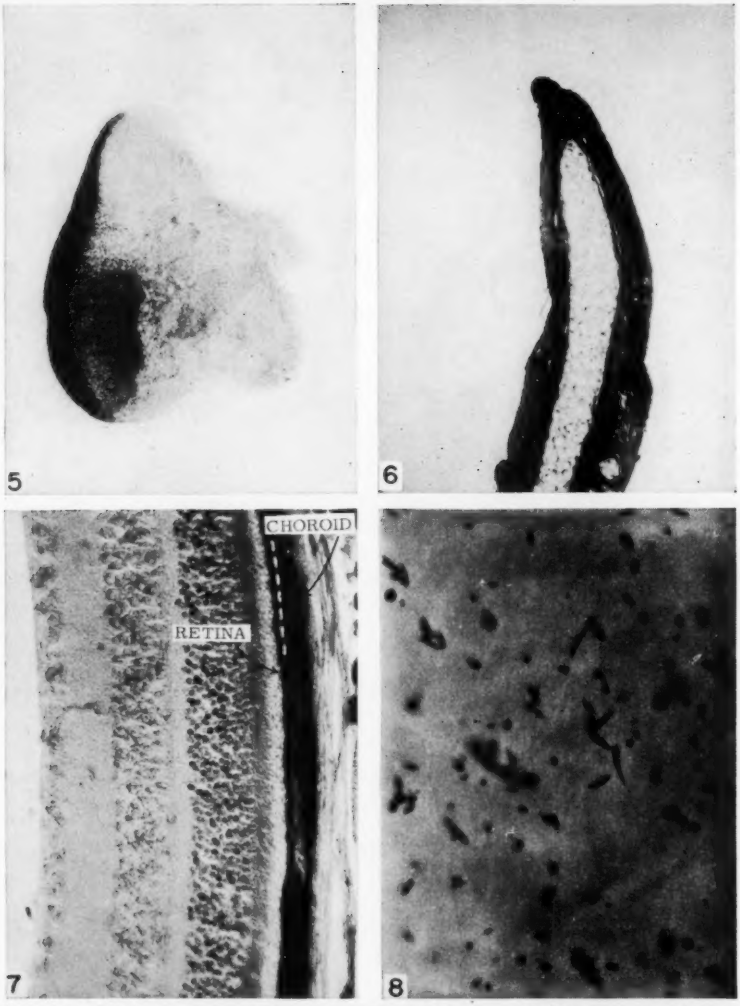


FIGURE 5.—Whole mount of unstained nictitans from a black mouse (*aa*). Note the densely packed melanocytes, particularly along the distal margin of the nictitans at the left. × 42.

FIGURE 6.—Cross section through the nictitans showing the cartilaginous core surrounded by dense connective tissue. Melanocytes are imbedded in the stratified epithelium covering the surface of the nictitans. × 120.

FIGURE 7.—Cross section through the eye of an *aaW*^{v}*w* mouse. The morphology and pigmentation of this eye are normal. Note the relatively thin pigmented layer of the retina and the much thicker pigmented choroidal coat of the eye. The dotted line indicates the area of separation of the choroid from the retina. The layers of the sensory retina extend nearly to the left margin of the photograph. × 290.

FIGURE 8.—Fresh, squash preparation of the retina and choroid from a 14 day old *aa* mouse. Note the great diversity in size and shape of the melanin granules. That this diversity is due to the retina is evident in figures 18 and 19. × 1490.

adjacent pigmented layer of the retina (fig. 7). In contrast to the choroid the pigmented retina is a true epithelium only one cell thick that arises early in embryonic life from the outer wall of the optic cup. Thus the pigmented cells of the retina have an embryonic history that decisively sets them apart from all other melanocytes of the body.

RESULTS

The occurrence and abundance of melanocytes in each of the six tissues studied in 50 genotypes of mice are presented in table 1. Table 2 summarizes the data on melanocyte morphology and on the shape and color of melanin granules in various genotypes as observed in the harderian gland.

Retinal melanocytes

The epidermal melanocytes composing the pigmented layer of the retina have the same embryonic history in all mice and the morphology of the cells—cuboidal epithelium—was not noticeably different in any of the genotypes examined, except

TABLE 2

Color and shape of melanin granules and melanocyte morphology as observed in the harderian glands of twenty-four basic genotypes

Genotype	Cell morphology	Granule morphology	Granule color
AA	NF[1]	ovoid	black
AAbb	NF	spheroid	brown
aa	NF	ovoid	black
aabb	NF	spheroid	brown
aabbc^{ch}c^{ch}	NF	spheroid	brown
A^{v}a	NF	ovoid	black
A^{v}abb	NF	spheroid	brown
AAc^{e}c^{e}	NF	ovoid	black
aadd	NP[2]	ovoid	black
A^{v}add	NP	ovoid	black
aabbdd	NP	spheroid	brown
aabbln ln	NP	spheroid	brown
aabbln lndd	NP	spheroid	brown
aaSl sl	NF	ovoid	black
aaW^{v}w	NF	ovoid	black
A^{t}A^{t}gl gl	NF	ovoid	black
aaru ru	NF	spheroid	brown-black
aatp tp	NF	ovoid	black
aaVa va	NF	ovoid	black
aabbVa va	NF	spheroid	brown
aaB^{lt}B^{lt}	NF	spheroid	brown-black
aapp[3]	—	shred-like	black
aaTo to	NF	ovoid	black
aaMi^{wh} Mi[3]	—	ovoid	black

[1] NF = nucleofugal (typical melanocyte with large, extended dendrites).

[2] NP = nucleopetal (melanocyte with enlarged perikaryon and reduced dendrites).

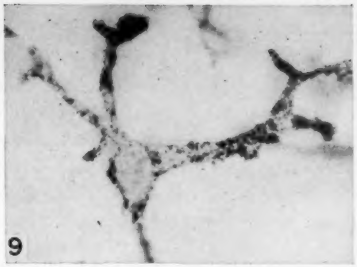
[3] Melanocytes not examined and granules observed only in hair shaft.

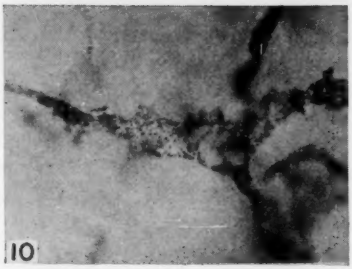
for those with morphologically altered eyes. The eyeless genotypes produced no eyes and consequently no pigmented retinas. The grossly deficient eyes of dominant white homozygotes had incomplete retinas—in which, however, the retinal cells, when present, retained a typical cuboidal morphology.

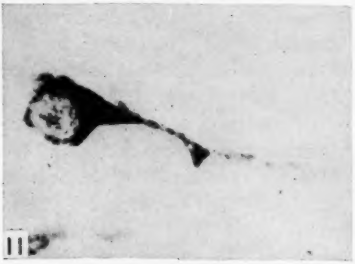
The capacity to form pigment and the characteristics of the pigment granules in the retina were greatly affected by genetic constitution. Albino animals (*cc*) of course produced no pigment even though retinal morphology was normal. The eyes of pink eyed (*pp*), dominant white ($Mi^{wh}Mi^{wh}$), and ruby eyed (*ru ru*) mice were characterized by reduced retinal pigmentation, pink eye showing the greatest reduction and ruby eye the least. Except for ruby eye all the genotypes produced either black or brown granules in accordance with the alleles present at the *B* locus (*bb* = brown, *Bb* and *BB* = black). Ruby eye granules from animals of black genotype (*aa ru ru BB*) were only slightly darker than typical brown granules and were thus intermediate between typical blacks and browns. Perhaps the most distinctive characteristic of retinal pigment granules was their striking diversity of size and shape (fig. 8) in all genotypes. Shape varied from spheres through all gradations to thin elongated rods, and in size the largest granules were at least five hundred times the volume of the smallest. This wide variation in shape and size contrasts sharply with the uniformity of granule size and morphology found in all dendritic melanocytes. In fact, the adjacent choroidal and retinal pigmented layers of the eye can readily be distinguished solely on the basis of the conspicuous differences in melanin granule morphology. These differences, occurring in adjacent cells of identical genotypes, can plausibly be attributed to the quite different embryological histories of the two types of melanocytes—one derived from the optic cup and the other from the neural crest. It is interesting to note that ruby eye genes not only reduce the number of melanin granules in the retina but also reduce both the average size and the range of diversity in granule morphology. No other gene was found that produced this effect.

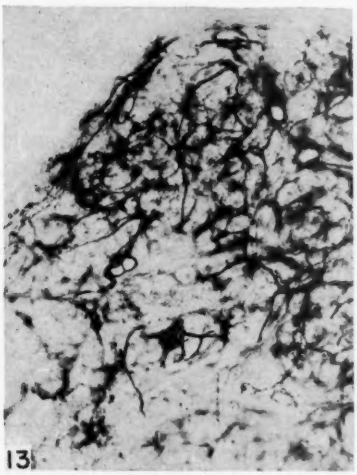
Dendritic melanocyte morphology

The dendritic melanocytes exhibit two distinct types of morphology and numerous variations in melanin granule characteristics. The commonest type of melanocyte has long relatively thick processes containing a substantial portion of the cell substance (figs. 9 and 10). The presence of the genes dilute (*dd*) or leaden (*ln ln*) results in a melanocyte (figs. 11 and 12) with fewer and thinner dendritic processes; in these cells the melanin granules are necessarily more concentrated in the main body of the cell. This type of melanocyte might be described as nucleopetal as contrasted with the commoner nucleofugal type (figs. 9 and 10). Dilute and leaden genotypes have a total volume of pigment that is at least as great as their corresponding non-dilute or non-leaden genotypes (RUSSELL 1948). The gross effect of these genes is to concentrate melanin granules into large clumps (compare figs. 13 and 14) thus reducing light absorption to give a "dilute" phenotype (RUSSELL 1946, 1948, 1949a, 1949b). These cells have been spoken of as containing "clumped" pigment granules (SILVERS 1953), but it should be noted that the granules are clumped or concentrated around the nucleus because the shape of the cell permits no other arrangement. The nucleopetal morphology probably results in an uneven release of granules from the









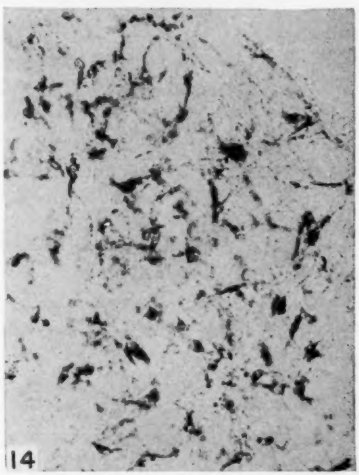


FIGURE 9.—Melanocyte from the harderian gland of a ten day old *aa* mouse. Note the distribution of melanin in thick dendritic processes. The black granules are ovoid. × 650.

FIGURE 10.—Melanocyte from the harderian gland of a ten day old *aabb* mouse. Note the thick dendritic processes and the spheroidal granules (brown). × 650.

FIGURE 11.—Melanocyte from the harderian gland of a four day old *aadd* mouse. Note the reduction in dendritic processes as compared to figures 9 and 10. The granules are ovoid and are clustered around the nucleus (nucleopetal type). × 650.

FIGURE 12.—Three melanocytes from the harderian gland of a six day old *aabbdd* mouse. Note the nucleopetal morphology and the spheroidal granules of the melanocytes. × 650.

FIGURE 13.—Melanocytes (nucleofugal) imbedded in the connective tissue capsule of the harderian gland of a ten day old *aa* mouse. Compare with figure 14. × 130.

FIGURE 14.—Melanocytes (nucleopetal) imbedded in the connective tissue capsule of the harderian gland of a four day old $A^{v}add$ mouse. Note the dilution effect produced by the concentration of melanin around the nuclei of the melanocytes. Compare with figure 13. × 130.

melanocyte to the epidermal cells of the hair bulb. Since the dendrites are the channels through which the granules are released, the reduction in size and number of these dendrites in *dd* or *ln ln* genotypes would seem to interfere with the release of granules to the epidermal cells; such interference could account for the irregular distribution of the granules along the hair (RUSSELL 1949b). At least some of the distinctive clumping of granules may result from the eventual disintegration of melanocytes which would release large masses of melanin granules at one time.

Observation of melanocyte morphology in any one genotype can not reveal whether the particular morphology is due primarily to the intrinsic genetic makeup of the pigment cell or to the cellular environment. However, melanoblasts of *dd* genotype that invaded transplanted tissue of *DD* genetic makeup produced melanocytes with a nucleopetal (*dd*) phenotype (REED 1938; REED and HENDERSON 1940). Thus this morphological characteristic of the melanocyte is due to its intrinsic genetic makeup. The morphology and color of melanin granules is not affected by changes at the *d* and *ln* loci, and no additive effect of the genes at these two loci has been observed.

Pigment granule morphology and color; black and brown

Reference has already been made to the variability of granule morphology in the retina of all genotypes. There is also a wide range of granule size in hair although a corresponding range of granule size is not evident in the melanocytes of the hair bulbs. It must be, therefore, that the granules continue to increase in size after they have left the melanocyte and been deposited in the epidermal cells of the hair. Within the dendritic melanocytes themselves, however, granules are relatively uniform in size, shape, and color. This is particularly evident in the melanocytes of the harderian gland. All the genotypes examined, except ruby eye (*ru ru*) and light (B^{lt} B^{lt}), had distinctly brown or black colored granules in the melanocytes of the harderian gland in accord with their genetic constitution. Black granules were typically ovoid in shape and brown granules were spheres (compare figs. 9 and 10). However, ruby eye granules were spheroidal in mice of black genotype. In such mice the change in granule morphology from ovoid to spheroidal is associated with a change in color from black to dark brown. Whether color and shape are interdependent characteristics is difficult to say in this case. A valid generalization emerging from this study, however, is that no fixed relationship exists between the color and shape of melanin granules (cf. RUSSELL 1949a). Brown, black, and yellow granules exist in many different shapes and also in many sizes. This is obvious (for black and brown) in the retina (fig. 8) but it is also evident in the hair.

Yellow melanin

The differentiation of yellow granules presents a clear illustration of the importance of the cellular environment in directing the course of melanoblast differentiation. Since the formation of yellow hair is due to the gene A^y at the agouti locus, all mice carrying this gene also carry genes for black or brown at the *B* locus. An examination of the six tissues previously listed shows that in yellow mice only the melanocytes of the hair bulbs synthesize yellow pigment. Melanocytes located in the remaining five tissue environments synthesize either black or brown pigment in accord with

their genetic constitution at the *B* locus. Thus the alleles A^v, A^l, A, and a^t at the agouti locus must act through the epidermal cells of the hair bulb to establish a follicular environment that directs the differentiation of melanoblasts to become yellow pigment producing cells. When the follicular cells contain the genes, *aa*, they fail almost completely to stimulate the differentiation of yellow melanocytes, although yellow hairs are frequently present on the ears, genitalia, and around the mammae. Thus some tissues, even of *aa* genotype, can provide an environment in which melanoblasts become yellow melanocytes.

SILVERS and RUSSELL (1955) have shown that melanoblasts of *aa* genotype produce yellow pigment when they invade $A^vac^sc^s$ (white) hair follicles. Likewise A^va melanoblasts produce black pigment when incorporated into *aa* hair follicles. Therefore, the agouti locus constitution of a melanoblast is not decisive in the differentiation of either black or yellow melanin. Further evidence of this is the occurrence of black (or brown in *bb* animals) pigment in A^va melanoblasts occurring in those tissue environments, other than the hair follicle, which stimulate melanogenesis. The intrinsic capacity of the melanoblast of, for example, A^vaBB genotype is apparently to produce black pigment as shown by the fact that such mouse melanoblasts transplanted to the coelom of white leghorn chicks there develop into black melanocytes (SILVERS unpublished).

Evidence has also been obtained demonstrating that the agouti locus plays a role within the melanoblast in determining the response of the pigment cell to local tract differences and general tract differences (REED and HENDERSON 1940; SILVERS and RUSSELL 1955). Further evidence for this intrinsic effect of the agouti locus may be deduced from the fact that the population of black pigment cells in *aaBB* ear skin is more numerous than in A^vaBB ear skin.

In addition to the A^v gene the recessive alleles at the gray lethal locus (*gl gl*) also appear to control the synthesis of yellow melanin through the mediation of the epidermal follicular cells. Agouti gray lethal animals produce hairs with obviously reduced pigment in the subterminal yellow band but with black pigment present in normal amounts in other regions of the hair. Furthermore, a large part of the yellow pigment that is present is distributed in clumps somewhat like the distribution found in dilute leaden animals. Melanocytes are unable to carry on a high level of yellow pigment production and are unable to release their pigment in the same manner as melanocytes producing black pigment. Gray lethal melanocytes in all pigmented tissues except the hair bulbs produce characteristic black granules only. Thus the gray lethal gene appears to act within the epidermal cells of the hair bulb in the presence of the A^v, A^l, A, and a^t genes to weaken and render abnormal the stimulus for yellow melanin production.

Genotypes without dendritic melanocytes

Of the 50 genotypes listed in table 1, 35 can induce the differentiation of melanocytes in each of the six selected tissues. Except for a few genotypes (indicated in table 1 by double symbols) there is remarkably little variation between animals of the same genotype in the pigment characteristics of these six tissues. In other tissues such as the meninges, thymus, etc., melanocytes were observed to be present irregu-

larly even in inbred strains of mice. This variability may indicate extreme sensitivity to minor fluctuations in embryonic history or to residual genetic differences in the inbred lines. In any event, melanogenesis occurs in at least one of the selected six tissues in all of the genotypes studied except albino (*cc*). Albino animals lack the enzymatic mechanism required for melanin synthesis. In three genotypes ($Mi^{wh}Mi^{wh}$, WW, WW^v) melanin is restricted to the retina and in a fourth, W^vW^v, pigmentation is usually restricted to the retina. These genotypes, particularly the first three, therefore do not differentiate any dendritic melanocytes. This absence of dendritic melanocytes cannot be due to any genetic incapacity to synthesize melanin for melanogenesis occurs in the retina of these animals. Since W^vW^v animals do occasionally produce dendritic melanocytes in ear skin or in the eyelid or in the harderian gland, it seems most plausible to attribute the lack of melanocytes in the tissues to the tissues themselves—that is, melanoblasts probably migrate out in normal fashion from the neural crest but except for occasional areas in W^vW^v animals the melanoblasts never find a cellular environment in which they can differentiate into mature melanocytes. Definite conclusions must await experimental transfer of presumptive melanoblasts of these genotypes to tissue environments that are known to be genetically favorable for melanoblast differentiation.

Melanoblast differentiation and the cellular environment

In twelve genotypes melanocytes regularly differentiate in at least one tissue in addition to the retina, but fail to differentiate in one or more of the remaining tissues. Melanoblasts of these genotypes obviously have the capacity to become fully mature melanocytes. However, some of the tissue environments are rendered unsuitable by their genetic makeup for melanocyte differentiation. The fact that in a single animal some of these five tissues provide a suitable environment for melanoblast differentiation while others do not emphasizes the fact that both the intrinsic genetic constitution and the right embryonic history are essential to a tissue in elaborating an environment that will stimulate melanoblasts to become melanocytes.

Alleles at the *C* locus provide a good example of action within the melanoblast. Melanoblasts of *cc* genotype produce no tyrosinase (FOSTER 1951) and therefore no melanin in any tissue environment. The extreme dilution alleles c^ec^e diminish the amount of pigment deposited on each granule—the gross effect being therefore to produce more lightly pigmented melanocytes in every tissue in which they differentiate. However, in hair bulbs of A^y genotype melanoblasts containing the genes c^ec^e are unable to synthesize yellow pigment granules and consequently the hair of $A^yac^ec^e$ animals is white, although the retina, choroid, harderian gland, etc. contain melanocytes with light black granules.

The genes *Sl* and W^v apparently interacted to depress the differentiation of melanoblasts in the nictitans and in the harderian gland as well. The combined effects of these genes can be regarded as an enhancement of spotting since about half of the harderian glands examined (10) contained no melanocytes and those containing pigment were frequently spotted.

The nictitans appears to provide the least favorable environment (of the six selected tissues) for pigment formation since it is the most sensitive to adverse genetic

changes. Two genotypes reduce or prevent pigmentation of the nictitans alone. These are varitint (*Va va*) which prevents, and taupe (*tp tp*) which drastically reduces, melanoblast differentiation in the nictitans. Leaden (*ln ln*) is also associated with a noticeable reduction in nictitans pigmentation, and dilute (*dd*) may likewise have a specific effect on the nictitans in combination with A^v or with *ln ln*.

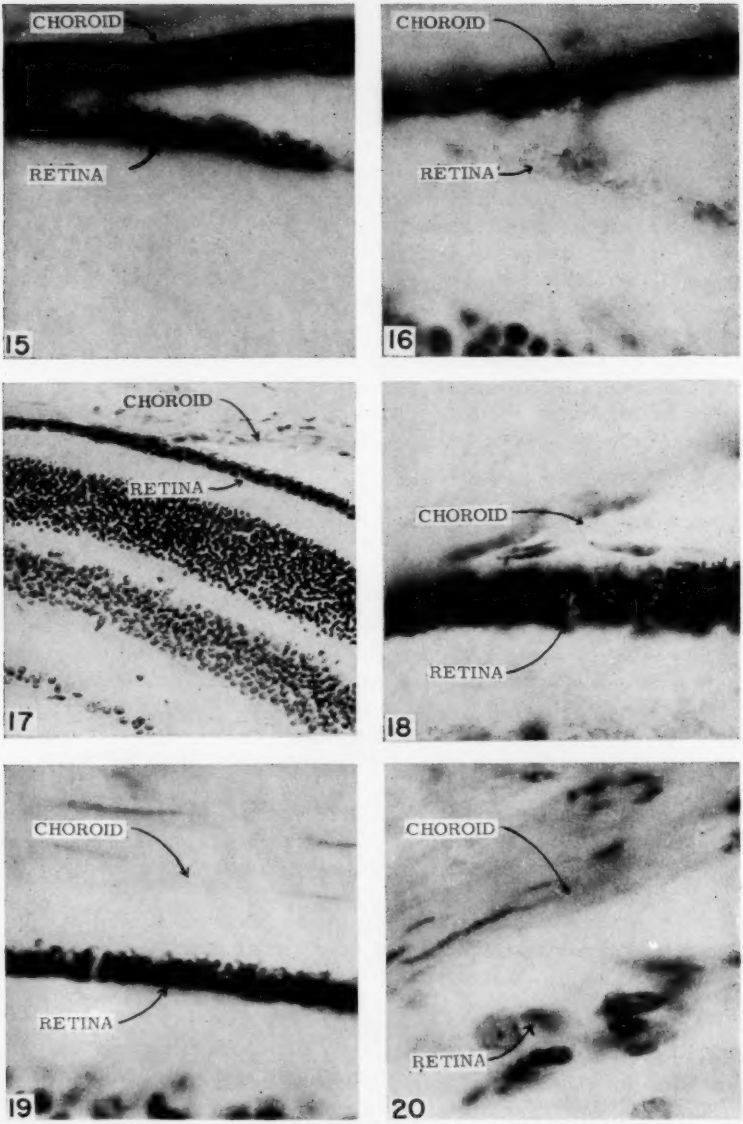
Four loci affected the pigmentation of the choroid—Mi^{wh}, *W*, *p*, and *s*. Of these Mi^{wh} was the most potent since even in heterozygous condition it rendered the choroid completely unsuitable for melanoblast differentiation. In homozygous condition the *W* or W^v alleles also prevented all choroidal pigment. The genes for piebald spotting have previously been reported as affecting choroidal pigmentation (GATES 1926; DUNN and MOHR 1952). In the present investigation the effects of piebald spotting on the choroid were found to be exceedingly variable. Some eyes exhibited almost normal choroids with only an occasional white spot while others contained an almost pigmentless choroid. Genotypes of *Ww ss* constitution produced eyes with no detectable choroidal pigment; apparently this effect is due to an interaction between *ss* and *Ww* that enhances spotting in the choroid as it does in the hair. Pink eye (*pp*) reduced both retinal and choroidal pigmentation; choroidal pigment was restricted to the region of the iris and only small amounts were found there. A comparison of these various genic effects on eye pigmentation can readily be appreciated by examining sections through the eyes as shown in figures 15, 16, 17, 18, 19, and 20.

Vital staining

In the presentation of the results of this investigation the point of view has been preferred that in all the genotypes examined (except *cc*) melanoblasts capable of forming melanin were present in each of the six selected tissues. Whether the melanoblasts differentiated into pigmented melanocytes then depended upon the kind of environment provided by the surrounding cells. Alternative views are possible and a common one is that melanoblasts are not present in the tissues that fail to become pigmented. To test these points of view efforts were made to stain melanoblasts or pigmentless melanocytes supravitally with brilliant cresyl blue and also with dopa. The harderian gland and ear skin of albino, black, and of spotted animals of various ages were stained with brilliant cresyl blue according to the procedure outlined by REYNOLDS (1954).

The dopa stained only those cells that had already begun to synthesize melanin and thus failed to reveal the presence of melanoblasts in any of the tissues examined. The results with brilliant cresyl blue were more variable and more difficult to interpret.

The ear skin of *aaBB* mice (C57 BL/6) at birth and at one, two, three, and four days of age was stained with brilliant cresyl blue. The ears of these mice at birth should have contained approximately equal concentrations of pigment cells either as melanoblasts or melanocytes. With increasing age the melanoblasts should have changed into melanocytes. Thus a melanoblast stain would reveal larger numbers of cells in the younger mice and in older animals the decrease in melanoblasts would be paralleled by a proportionate increase in visible melanocytes. Although melano-


CHOROID
RETINA
15
CHOROID
RETINA
16
CHOROID
RETINA
17
CHOROID
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18
CHOROID
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19
CHOROID
RETINA
20

FIGS. 15–20

cytes with only a few melanin granules did stain selectively with the brilliant cresyl blue the younger melanoblasts which must have been present in younger ears did not stain. In the ear skin of older animals (albino, black, or spotted) a variety of cell types took up the brilliant cresyl blue stain. A common type of cell revealed by the stain so closely resembled melanocytes in morphology and in the size and distribution of their blue stained granules that, except for the color of their granules, they could not be distinguished in appearance from mature melanocytes. However, because of the results obtained with the skin of newborn mice, these cells can scarcely be embryonic melanoblasts. Such cells may, however, be pigmentless melanocytes—that is, melanoblasts that have differentiated into mature type cells without the formation of melanin granules (see BILLINGHAM and MEDAWAR 1953). The lack of specificity of the stain makes the results merely suggestive, and tentative conclusions based on staining reactions cannot be accepted without support from other evidence. With these limitations in mind the evidence from staining lends tenuous support to the belief that melanoblasts of all genotypes invade these six tissues. Where the cellular environment proves unfavorable for their normal maturation their differentiation is diverted towards a type of maturity that does not involve melanogenesis—that is, a pigmentless melanocyte which stains with brilliant cresyl blue. The truly embryonic melanoblast will not however stain with the brilliant cresyl blue.

DISCUSSION

Tissue environments

The hypothesis of melanoblast differentiation that is favored in this investigation places the primary responsibility for the differentiation directly upon the cellular environment of the melanoblast. This hypothesis is preferred because it is the most economical and because some evidence directly supports it. On the basis of present evidence it may be assumed that a melanoblast possesses at most two metabolic systems by which it responds to melanogenic stimuli from the cell environment. Hair follicles of A^y genotype activate the reaction system in melanoblasts that leads to yellow melanin. A^l, A, and a^t genotypes result in the production of some yellow

FIGURE 15.—Cross section through the normal retina and choroid of an *aabbdd* mouse. The photograph was taken of an area in which the choroid and retina became partly separated during sectioning. This figure serves as a standard for comparing with figures 16–20. × 1215.

FIGURE 16.–Cross section through retina and choroid of an *aaru ru* mouse. Note the great reduction in the pigmentation of the retina. × 1215.

FIGURE 17.—Cross section through retina and choroid of an $Mi^{wh}Mi$ mouse. Note the absence of choroidal pigmentation. × 220.

FIGURE 18.—Cross section through retina and choroid of an Mi^{wh} *Mi* mouse. This photograph was taken of the preparation shown in figure 17 to show in higher magnification the retina and choroid in the area where they have been partly separated. No choroidal pigmentation is present. × 1215.

FIGURE 19.—Cross section through retina and choroid of an $aaWW^v$ mouse. Note the normal retina and the absence of choroidal pigmentation. Variation in granule size and shape in the retina is also evident. × 1215.

FIGURE 20.—Cross section through retina and choroid of an *aapp* mouse. A very few melanin granules can be found in the retina and choroid of these animals although they are difficult to find and none are evident in this photograph. × 1215.

melanin in hair follicles. All other tissues (and follicles of *aa* genotype) that provide a melanogenic stimulus activate the metabolic system that leads to the formation of black or brown melanin in accord with melanoblast genotype. The simplest assumption is that all tissues that provide melanogenic environments (except follicles inducing yellow melanin formation) activate melanoblasts by essentially the same metabolic devices. For if these devices are not alike then melanoblasts must be equipped with a variety of reaction mechanisms—one for each type of environment in which they may reside. This is implausible in view of the large variety of tissue environments in which melanocytes occur. Thus we may assume (yellow melanin aside) that all melanoblasts are exposed to the same differentiating stimuli in all melanogenic tissue environments and that they respond by means of a single reaction system. The different tissues of the mouse either elaborate these stimuli or they do not. The capacity of a tissue to elicit melanogenesis depends upon the embryonic history of the tissue (that is, what kind of tissue it has become—nictitans, harderian gland, etc.) and upon the genetic composition of the tissue. For example, as the primordium of the harderian gland gradually matures into the adult tissue it simultaneously establishes (like an intrinsic by-product) a tissue environment in which melanoblasts will become melanocytes. However, if the harderian gland cells happen to contain the genes Mi^{wh} *Mi* then they do not elaborate an environment suitable for melanoblast differentiation, even though in other respects the harderian gland cells are normal. It is not just the presence of Mi^{wh} *Mi* genes that prevents formation of a melanogenic environment, because melanogenic environments are established by cells of this identical genotype in the hair bulb and in the ear skin of Mi^{wh} *Mi* mice. Thus the expression of genic activity is completely dependent upon the type of cell in which the gene activity occurs. Furthermore, cells of the same general type (follicular cells, for example) but located in different regions of the body may exhibit striking differences in their effects on adjacent cells. This is clearly shown by the work of REED (1938). This investigator transplanted skin from embryonic albino mice ($a^t a^t cc$) to the dorsal and ventral regions of black-and-tan mice ($a^t a^t$) and of dilute black-and-tan mice ($dd\ a^t a^t$). Melanoblasts from the host invaded the transplant and produced tan hairs or black hairs in conformity with the dorsal or ventral origin of the grafted skin and regardless of the location of the graft on the host. Ventral epidermis of albino genotypes provides an environment that will activate responsive melanoblasts ($a^t a^t$) to produce yellow ("tan") melanin. Grafted dorsal albino (*aacc*) epidermis stimulates differentiation of black melanocytes from either dilute or non-dilute hosts, but the morphology of the pigment cell remains autonomous—that is, melanocytes of dilute (nucleopetal) genotype impart a dilute type of pigmentation to hairs composed of epidermal albino cells of non-dilute genotype. Thus the expression of the black-and-tan gene in a melanoblast is dependent upon the particular tissue (dorsal or ventral epidermis) in which the melanoblast resides, while the expression of the gene for dilution is intrinsic to melanoblasts and autonomous with regard to the cellular environment.

Spotted pigmentation patterns

Spotted pigmentation patterns, though common in many kinds of animals, are among the most perplexing to interpret. RAWLES (1947) demonstrated that mouse

pigment cells arise from the neural crest. These migratory melanoblasts invade many tissues in some of which they synthesize melanin. It can be assumed therefore, that a white spot is due either to the absence of melanoblasts or to their failure to differentiate in that area. Absence of melanoblasts might be attributed either to failure to migrate into the area or to their destruction after arrival and before the synthesis of pigment occurred. Several considerations can be brought to bear in evaluating these possibilities. First, the existence of islands of pigmented hairs surrounded by white areas seems inconsistent with any effective block to melanoblast migration. In this connection BILLINGHAM and MEDAWAR (1953) have identified cells in white spotted areas which appear to be derivatives of melanoblasts as judged by their distribution, morphology, and staining reactions. Secondly, skin from a white spot when transplanted to a pigmented area is invaded by host melanoblasts that deposit pigment in the previously white area (RAWLES 1953, 1955; SILVERS and RUSSELL 1955). If spotting is due to an exclusion or killing of melanoblasts by hostile tissues then such mechanisms must be transitory. Nevertheless, responsibility for white spotting does seem to reside within the skin (or other spotted tissue such as choroid or harderian gland) rather than within the melanoblast. Since melanoblasts throughout the body are probably alike and certainly so genetically, their failure to synthesize pigment must be due to lack of suitable stimuli. In passing it may be noted that any mechanism intrinsic to the melanoblasts that prevented on the average a certain fraction of them from maturing into melanocytes would result in a dilution effect rather than a spot. The mutant silver (*si si*) may indeed involve such a mechanism. According to DUNN and THIGPEN (1930) silvering is the result of a generalized reduction in the number of pigment granules. White, black, and mosaic hairs are intermingled all over the body to produce a silvering effect similar to that obtained by CHASE (1951) with X-radiation. Since a random destruction of melanoblasts probably forms the basis of the silvering effects of X-radiation, it seems plausible to ascribe hereditary silvering (*si si*) to a corresponding random genetic destruction of melanoblasts. In support of this hypothesis is the fact that a genetic destruction of melanoblasts is responsible for the white plumage of white leghorn chickens (HAMILTON 1940). Melanoblasts differentiate in white leghorn feather follicles but die before depositing noticeable quantities of melanin in the epidermal cells of the feather—hence the feathers are white.

White spotting in contrast to generalized loss of pigment is probably due to localized failures of the tissue environment to promote melanoblast maturation. Some evidence from tissue cultures of chick skin suggests this viewpoint. MARKERT (1948) found that in tissue culture chick dermis inhibits melanoblast differentiation while the epidermis is at least neutral and may promote melanoblast differentiation. The increased pigmentation of mouse skin after painting with turpentine and acetone (REYNOLDS 1954) may likewise be attributed to a disturbance of normal tissue activity resulting in the destruction of the inhibiting properties of the skin. The fact that migrating melanoblasts can pigment hair follicles of transplanted white spotted skin (SILVERS and RUSSELL 1955) suggests that disruption of the tissue at the margins of the graft might remove normal inhibiting properties of the follicles. However, the careful work of RAWLES (1955) disproves this hypothesis for the hooded rat. She demonstrated that transplantation alone does not result in pigmentation of white

spotted skin. An alternative, and more plausible hypothesis, attributes the absence of melanocytes in a white spot to the lack of an essential step in a sequence of differentiating stimuli. Melanoblasts that had developed past this blocked step in adjacent areas before migrating into the spotted follicles could then respond to the succeeding stimuli present in spotted skin and thus mature into melanocytes.

On the other hand, the analysis of a spotted mutant (splotch) of the mouse by AUERBACH (1954) tends to fix responsibility for pigment failure on the neural crest rather than upon the cell environment. AUERBACH implanted presumptive neural crest of *Sp Sp* embryos into the coelom of chick embryos. These implants yielded no pigment cells although corresponding transplants from splotch heterozygotes did produce pigment. Mice heterozygous for splotch have a white belly spot and white extremities; the homozygote dies at approximately 14 days in utero. By this time the retina is pigmented but the existence of dendritic melanoblasts can only be determined from transplants all of which have so far failed to produce pigment, even in a favorable cell environment. In many respects *Sp* resembles Mi^{wh}, *W*, and W^{v} in its effect on pigmentation. The heterozygotes all have white belly spots and the homozygotes are all essentially one big white spot except for the pigmented retina. Except for splotch, however, the neural crest development of these mutants is not grossly abnormal but subtle abnormalities of the neural crest in the homozygotes could prevent the origin of melanoblasts. Alternatively, homozygous tissues may not produce the stimuli required to bring about the maturation of melanoblasts. Since

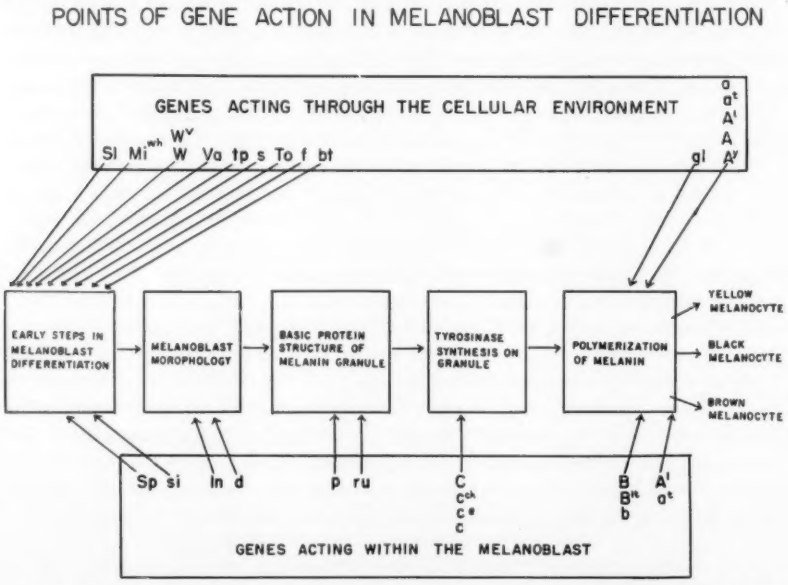
POINTS OF GENE ACTION IN MELANOBLAST DIFFERENTIATION
GENES ACTING THROUGH THE CELLULAR ENVIRONMENT
Sl Mi^wh W^v W Va tp s To f bt
a a^t A^l A A^v gl
EARLY STEPS IN MELANOBLAST DIFFERENTIATION
MELANOBLAST MORPHOLOGY
BASIC PROTEIN STRUCTURE OF MELANIN GRANULE
TYROSINASE SYNTHESIS ON GRANULE
POLYMERIZATION OF MELANIN
YELLOW MELANOCYTE
BLACK MELANOCYTE
BROWN MELANOCYTE
Sp si In d p ru C C^ch C^e c B B^w b A^l a^t
GENES ACTING WITHIN THE MELANOBLAST

FIGURE 21.—Probable times and places of gene action during melanoblast differentiation in the house mouse.

the cells of these mutants do have the genetic capacity to produce melanin as shown in the retina, the failure to do so in other tissues of the body must stem from abnormal embryonic relationships at some step in melanoblast differentiation. The problem is to discover the time and place during development at which the genes *Sp*, *Mi*^{wh}, and *W* act to prevent pigmentation.

Similar problems are presented by the mutants flexed tail (*ff*) and belted (*bt bt*) each of which is characterized by a variable spotted pigmentation pattern. Since the flexed tail genes, in particular, exert other significant effects on metabolism such as siderocytic anemia and tail flexure (KAMENOFF 1935; GRÜNEBERG 1942a, 1942b) it is probable that they at least act through the tissue environment in affecting melanoblast differentiation.

Place of gene action

It seems reasonable to consider melanoblast differentiation as proceeding through an orderly sequence of steps each of which is dependent upon the successful completion of the previous step. This stepwise differentiation offers many opportunities for genic intervention either by acting directly within the melanoblast or by altering the behavior of the cells composing the tissue environment.

In figure 21 an attempt is made to summarize the probable principal points of gene action on the sequence of steps leading to melanoblast differentiation. (A key to the gene symbols used is provided in table 3.) A similar attempt has been made by WRIGHT (1941) for the genes affecting pigmentation in the guinea pig. In the present scheme for the mouse the spotting genes—*Mi*^{wh}, *W*, *Va*, *s*, *f*, *bt* (and also *To*, *tp* and *Sl*)—are all represented as affecting the early steps in melanoblast differentiation by controlling the tissue environment. The genes at the agouti (*A*) and gray lethal (*gl*) loci likewise appear to act via the tissue environment but in this case on the terminal step of melanogenesis—that is on the step responsible for the

TABLE 3

A key to the gene symbols used in figure 21 and elsewhere in this report

Gene symbol	Name of gene	Gene symbol	Name of gene
a	non-agouti	*gl*	grey-lethal
A	agouti	*ln*	leaden
A^{l}	light-bellied agouti	*Mi*^{wh}	dominant white
a^{t}	black-and-tan	*p*	pink eye
A^{y}	yellow	*ru*	ruby
b	brown	*s*	piebald
B	black	*si*	silver
B^{lt}	light	*Sl*	steel
bt	belted	*Sp*	splotch
c	albino	*To*	tortoise shell
C	colored	*tp*	taupe
c^{ch}	chinchilla	*Va*	varitint waddler
c^{e}	extreme dilution	*W*	dominant spotting
d	dilution	*W*^{v}	viable dominant spotting
f	flexed-tail		

polymerization of melanin to yield the final color—yellow, brown, or black. The action of the genes at the remaining six loci can be interpreted as occurring within the melanoblasts themselves. Dilute (*dd*) and probably leaden (*ln ln*) control melanoblast morphology independently of the tissue environment (REED 1938). Pink eye (*pp*) (RUSSELL 1949a) and ruby eye (*ru ru*) both alter the shape of melanin granules and presumably therefore change the protein matrix of the granule within the melanocyte. The albino locus (*cc*) controls the enzymatic activity essential for melanogenesis and this enzyme, when present, is located within the melanoblasts themselves. Furthermore, albino tissue can support the differentiation of non-albino melanoblasts (REED 1938). Finally genes at the *B* locus affect melanin polymerization to determine the brown or black color of the granules. In contrast to yellow the brown or black color of any mouse appears to be independent of tissue environment and therefore is probably autonomously determined by the melanocyte. The fact that genes exist which independently affect black (e.g. *pp*) and yellow (e.g. *gl gl*) pigmentation makes improbable any linear sequence in the production of these pigments and strengthens the hypothesis that the alleles at the *A* and *B* loci control alternative paths in the polymerization of melanin.

Although these genes have been discussed as if they acted at only one time and place, it should be realized that many of them probably exert significant influences on the course of development by their metabolic activity in a variety of tissues at frequent intervals in development. In fact, the agouti locus appears to act both within the melanoblast and in the tissue environment to direct differentiation. Hence in figure 21 the alleles A^l and a^t are listed in two positions. Other genes may likewise deserve a double listing. Nevertheless, in this discussion an effort has been made to focus attention on what is believed to be the major contribution of the gene to melanoblast development.

The scheme of figure 21 is of course hypothetical but seems to be consistent with all the major facts known about the effects these genes have on pigment formation in the mouse. No such interpretation can be convincingly established by observation alone; experimental reassortment of melanoblasts and tissue environments will be necessary for a critical testing of the scheme. Fortunately such experimental interchanges are feasible and constitute the next step in gathering evidence with which to delineate the relative roles of genotype and embryonic history in the control of cellular differentiation.

SUMMARY

1. The occurrence, abundance, and characteristics of melanocytes in the tissues of the house mouse have been described. These characteristics have been related to the genotype and embryonic history of the melanocyte and to the genotype and differentiated characteristics of the following six tissues: nictitans, harderian gland, ear skin, hair follicle, choroid, and retina.

2. Melanoblast differentiation requires a favorable genotypic constitution and embryonic history in both the pigment cell and in the cells composing the tissue environment.

3. In general the color of melanin granules is related to their morphology—brown

granules are spheres and black granules are ovoids. However, since retinal melanocytes (either brown or black) produce granules of extremely diverse sizes and shapes, no necessary dependence can exist between granule shape and color.

4. Yellow melanin is formed by melanocytes only in the cellular environment of a hair follicle of appropriate genotype. All other melanogenic tissue environments induce the synthesis of either black or brown melanin in accord with the genes present in the melanocyte at the *B* locus.

5. Genes exist that affect the synthesis exclusively of black, brown, or yellow melanin, or of all three colors; these genetic effects render improbable any linear sequence among these pigments in the synthesis of any of them.

6. The nucleopetal (*dd*—dilute, or *ln ln*—leaden) or nucleofugal (*D-Ln-*) morphology of melanocytes is determined exclusively by their own genetic makeup independently of the genetic constitution of the cells in the tissue environment.

7. White spotting on pigmented animals may be interpreted as a failure of the tissue environment to elicit an essential step in melanoblast differentiation.

8. Neither brilliant cresyl blue nor dopa selectively stain melanoblasts although dendritic cells similar in appearance to melanocytes do stain with the brilliant cresyl blue.

9. A scheme is presented which summarizes the probable principal points of gene action (for 19 loci) on the sequence of steps leading to the differentiation of melanocytes.

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